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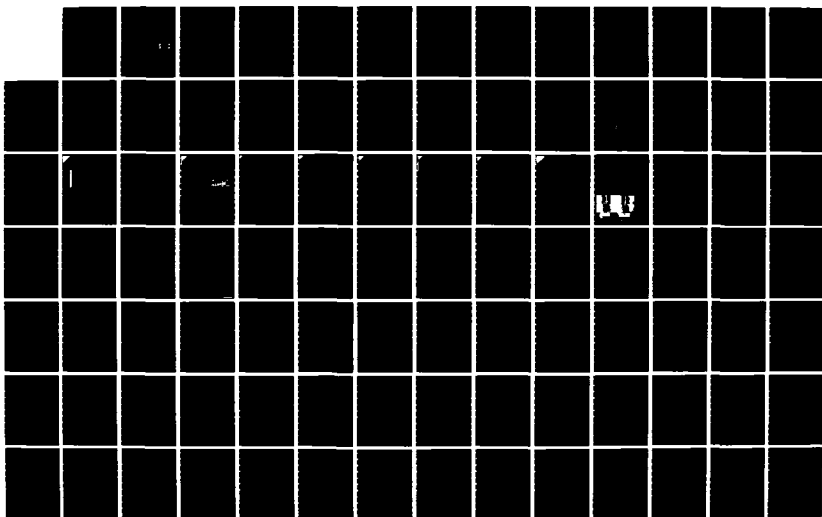
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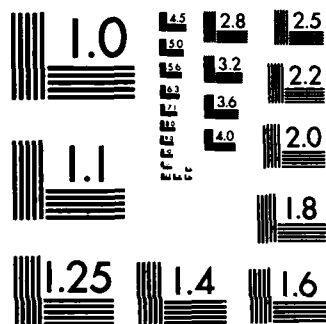
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OFFICE OF NAVAL RESEARCH

Contract N00014-79-C-0126

Annual Progress Report

October 1, 1982 - September 15, 1983

by

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Annual Progress Report
October 1, 1982 - September 15, 1983

"Molecular Mechanisms Involved in Tissue Swelling Due to Injury and Due to Exposure to Low Temperature and Massive Water and Electrolyte Loss in Diarrheal Disorders"

NO0014-79-C-0126

This annual progress report will follow past tradition and will be given in three sections: (Section I) a brief Background Information including much work we accomplished under ONR contract; (Section II) a summary of laboratory work; and (Section III) a summary of non-laboratory work.

I. Background Information

The basic unit of all life is the living cell. A sound knowledge of the physico-chemical nature of the living cell may well be the key to all successful biomedical investigations of the future.

This is not to say that biomedical research cannot produce useful results without a correct concept of the living cell. It is only that, in the long run, much time, efforts, and money may be wasted if such research is built on an incorrect concept of the living cell in the same way that a house beautifully designed and constructed may eventually prove to cost much wasted time, efforts, and money, if the house is built on shifting sand.

In theory, the way to cope with this problem of locating a correct foundation for further investigation is simple enough. One looks for and finds solid ground before starting to build. In reality, the house was always urgently needed and construction might have proceeded before there was no doubt that what appeared to be solid ground was in fact otherwise. Once construction has begun, there will be enormous pressure to ignore evidence that the foundation is not sound. So when clear-cut evidence of sandy foundation does begin to surface, it would take great leadership for those in command to bring about the needed change of course thereby forestalling impending catastrophe.

Let us now return to biomedical research. There has been irrefutable evidence more than twenty years ago that the basic theory of the living cells, the membrane-pump theory, taught as truth in virtually all textbooks from high school up and used directly or indirectly as the foundation of all biomedical research, is wrong.

The evidence against the membrane-pump theory is simple: One of the first proposed pumps, the Na pump, would (under certain rigorously controlled conditions) require from 15 to 30 times as much energy than the total energy available to the cell. Furthermore, the Na pump is only one of many pumps that have been formally proposed and which numbered more than twenty already as counted in 1968. These were limited to pumps postulated to exist in the plasma membrane surrounding the cells. Many more pumps must also be (and were) postulated to exist at the membranes of subcellular particles. The much larger membrane surface areas of these subcellular particles than the plasma membrane demand proportionately more energy for pumping. As an example, the total surface of

liver mitochondria is twenty times larger than that of the plasma membrane and would thus require that much more energy for every pump than the corresponding plasma-membrane pump. To the best of my knowledge, no one has publicly contested the serious difficulty revealed; nor has anyone come forward with a feasible, meaningful overall energy balance sheet. In the meanwhile, the proponents of the membrane-pump theory have continued to act as if these serious problems facing the theory never happened. To show how bad the situation has become, I cite the following well-documented specific case.

In the past decades, Cambridge, England, had become the "Mecca" of the membrane-pump theory because the Cambridge University has produced some of the world's greatest scientists. In 1975 two scientists from the Physiological Department of the Cambridge University, Glynn and Karlsh published in the Annual Review of Physiology, a first-of-its-kind review on the "Na Pump." This article reviewed the subject covering a total of 235 references published over a period of 22 years during which most of my work had been published. Yet there was something not quite right in this review. Professor H. R. Catchpool of the University of Chicago commented on this review thusly in Perspectives in Biology and Medicine 24:164 (1981): "The first comprehensive review which mentioned the Na pump in the title was that of Glynn and Karlsh... Glynn and Karlsh listed 235 articles in support of the sodium pump and none opposed. Yet Ling's ideas had been around for 25 years, so had ours; so has Troschins..."

How seriously these carefully executed omissions by scientists like these (whose judgments were trusted by virtually all other scientists) had violated the basic code of scientific ethics can be evaluated in the context of the words of an earlier English scientist-mathematician of renown, Charles Babbage of the last 19th century. Babbage pointed out that scientific fraud was not limited to data fabrication. Two other kinds of fraud exist, The third kind of fraud was called "cooking" defined by Babbage as "the choosing of those data that fitted the researcher's hypothesis and the discarding of those that did not..." (see Morton Hunt, New York Times, November 1, 1981.)

There have also been in recent times better publicized case after case of other types of scientific fraud, "fabrications" by scientists at the best teaching and research institutions, including Sloan-Kettering, Columbia, Cornell, Yale, and Harvard, all in the realm of biomedical research. And one outstanding case also directly involved the Na-pump (Spector at Cornell, see Betrayers of the Truth (Broad and Wade, Simon and Schuster, 1983). Perhaps Spector, a very bright, young scientist, was quite aware of the quicksand they stood on and the little real hope for true progress to be made on this foundation.

In the early 50's, alarmed by the energy imbalance situation mentioned above, I went to seek an alternative physico-chemical model of the living cells. This search culminated in the publication first in 1952 briefly and in detail in 1962 of a new theory called the association-induction hypothesis. In this theory, levels of ions and other solutes in the living cells are not maintained by the activities of energy-consuming pumps but reflect a non-energy consuming equilibrium state. Thus, the high level of K^+ in the cell is not due to constant inward pumping but the result of selective adsorption on many anionic sites of cellular proteins. The low level of Na^+ is not due to constant outward pumping but reflects the existence of cell water in a physical state different from that of normal liquid water, i.e., the state of polarized multilayers. Na^+ sugars,

free amino acid as well as other large and complex molecules found in low concentration in the cell are (partially) excluded because they have lower solubility in the cell water existing in the state of polarized multilayers. (Virtually all of these basic concepts have by now been experimentally confirmed, see below.)

One would hope that subscribers to the membrane-pump theory would join us in designing experiments to test the alternative theories - as scientists should do. But this was not what happened. Nor was it the first time that intelligent people should behave like ostriches and defended the membrane pump theory more like religious fanatics than objective scientists. Therefore the kind of "cooking" Glynn and Karlsh and others like them did had far-reaching consequences. As a result, it became harder and harder for scientists like myself to survive who foresaw all too clearly the impending danger of "quicksand" and tried to warn others. Despite these difficulties, I did last this long. What has made this survival possible will be made clear in the following verbatim quotation of the opening paragraph of the "Acknowledgment," of my forthcoming book (see below).

"The Office of Naval Research supported my work between 1953 and 1957 through a contract award to Professor R. W. Gerard (ONR 110 128). Later after I settled down at the Pennsylvania Hospital, ONR continued its funding and it has, without a single interruption, supported my work for the last 18 years under Contract N00014-71C-0178. In particular I take pleasure in thanking Dr. Arthur B. Callahan, who had both the scientific insight and the courage to support my work through the years when many considered it highly controversial."

I want to bring this out because I feel that in the last twenty years the Office of Naval Research in general and Dr. Arthur B. Callahan (who has now retired) in particular, have permitted a major branch of biomedical science to live on by supporting a small minority of dissenting scientists from oblivion by supporting the "Water and Ion Program." And I am equally certain that they had actually allowed us to build a more solid foundation to future biomedical research than the quicksand of more and more membrane pumps. In support of this view, I also pointed out in my forthcoming book the following:

"It gives me great pleasure that the support of the ONR, the NIH, the Hartford Foundation, Pennsylvania Hospital, and my friends has already borne practical fruits,* directly benefiting mankind.

* NMR scanning, which allows continued quantitative investigations and monitoring of normal and diseased human body parts without surgery or X-ray irradiation, was invented by Dr. Raymond Damadian, the patent holder, who wrote me on November 9, 1977:

'On the morning of July 3, 1977, at 4:45 A.M.... we achieved with great jubilation the world's first NMR image of the live human body. The achievement originated in the modern concepts of salt water biophysics [introduced by] your treatise, the association-induction hypothesis.' "

In a few month's time a book telling the whole history of cell physiology - including why at one time the membrane-pump theory became widely accepted and the results of thirty years' of experimental testing of the membrane theory

and the AI hypothesis under the title, "In Search of the Physical Basis of Life," Plenum Publishing Corp., New York, early 1984.

II. Laboratory Work

Our research continues in the direction of understanding the physical state of water and ions in living cells as these are the essential foundation knowledge for all future biomedical research and in applying this knowledge to solving other practical problems of interest to the Navy. *Subjects discussed are:*

(A) The Rotational Correlation Time of "Non-solvent" Water,

The discovery of the condition that creates in vitro water existing in the state of polarized multilayers and the extensive NMR investigation of the relaxation behavior of this water has led to an important conclusion: Water which has no solubility for probe molecules like Na citrate has a rotational correlation time lengthened merely by a factor of 3 to 10, rather than by a factor of thousands or millions if water were truly ice-like. This finding permits conciliation of (1) the modest reduction of the rate of water diffusion in living cells (by a factor of 2), (2) the modest reduction of T_1 values of water protons in living cells, and (3) a non-energy consuming explanation of the low level of Na^+ according to the AI hypothesis. (For details, see enclosed manuscript, MS # 4.

(B) The Establishment of the Adsorbed State of Counterions (Na^+) in Ion Exchange Resins,

With highly selective Na^+ electrode, we established that virtually all the counteraction in nuclear-sulfonate ion exchange resin exists in a one cation-one site close contact adsorbed state. How this disproves the textbook interpretation of ion exchange resin selectivity and the interpretation of ^{23}Na NMR data are fully explained in the enclosed MS #9.

(C) The Dependence on Ca^{++} Concentration in Cell Swelling Induced by Isotonic KCl in Muscle Cells Without an Intact Cell Membrane,

Swelling of frog sartorius muscles cut into 2 mm and 4 mm segments with no intact membrane in 0.1 M KCl depends strongly on Ca^{++} concentration in the medium. At high Ca^{++} concentration (1 mM) or in the presence of no Ca^{++} but 1 mM EDTA, there was minimal swelling. Maximum swelling occurred at a Ca^{++} concentration of 0.01 to 0.1 mM. The data closely parallel similar observation by Edelmann, who observed Ca^{++} -dependent K^+ uptake in extremely thin sections (0.2 μ) of frozen dried, imbedded frog muscle cells. Both sets of data agree with the AI hypothesis that selective K^+ adsorption depends on the Ca^{++} acting as a cardinal adsorbent

III. Non-laboratory Work

A. As mentioned above, the book, "In Search of the Physical Basis of Life" is now at the page-proof stage. Aside from its many unique features, the book presents more than 40 portraits of scientists who have made significant contribution to man's search for the physical basis of life. The book is due to appear in early 1984.

B. Additional Work

Published

- 1 Ling, G. N., and Murphy, R. C., "NMR Relaxation of Water Protons Under the Influence of Proteins and Other Linear Polymers", Physiol. Chem. Phys. 14:209 (1982)
- 2 Ling, G. N., and Murphy, R. C., "Apparent Similarity in Protein Compositions of Maximally Deviated Cancer Cells", Preliminary Note, Physiol. Chem. Phys. 14:213 (1982)

In Print

- 3 Ling, G. N., and Ochsenfeld, M. M., "Studies on the Physical State of Water in Living Cells and Model Systems. I. The Quantitative Relationship Between the Concentration of Gelatin and Certain Oxygen-Containing Polymers and Their Influence Upon the Solubility of Water for Na^+ Salts", Physiol. Chem. Phys. 15:xxx (1983)
- 4 Ling, G. N., and Murphy, R. C., "Studies on the Physical State of Water in Living Cells and Model Systems. II. NMR Relaxation Times of Water Protons in Aqueous Solutions of Gelatin and Oxygen-Containing Polymers Which Reduce the Solvency of Water for Na^+ , Sugars, and Free Amino Acids", Physiol. Chem. Phys. 15:xxx (1983)
- 5 Ling, G. N., "Studies on the Physical State of Water in Living Cells and Model Systems. III. The High Osmotic Activities of Aqueous Solutions of Gelatin, Polyvinylpyrrolidone and Poly(ethylene oxide) and Their Relation to the Reduced Solubility for Na^+ , Sugars, and Free Amino Acids", Physiol. Chem. Phys. 15:xxx (1983)
- 6 Ling, G. N., "The Association-Induction Hypothesis. A Theoretical Foundation Provided for the Possible Beneficial Effects of a Low Na, High K Diet and Other Similar Regimens in the Treatment of Patients Suffering from Debilitating Illnesses", Symp. on Metabolic Dysfunctions, Mexico, Jan 30-31, 1981, Agressologie 24:xxx (1983)
- 7 Ling, G. N., "The Molecular Mechanisms of Cellular Potentials", in Structure and Function in Excitable Cells, Plenum Publishing Corp., New York
- 8 Ling, G. N., "Experimental Confirmation of the Polarized Multilayer Theory of Cell Water Including Data That Lead to An Improved Definition of Colloids", in New Trends in the Study of Water and Ions in Biological Systems, Plenum Publishing Corp., New York

Manuscripts

- 9 Ling, G. N., and Zhang, Zheng lian, "Evidence Showing That the Bulk of Na^+ in Sulfonate Ion Exchange Resin Exists in an Adsorbed State and Its Significance for the Interpretation of NMR Data on Na^+ , K^+ , and Other Ions in Living Cells", Physiol. Chem. Phys.
- 10 Ling, G. N., and Kwon, Y., "Cold Injury-Induced Brain and Other Tissue Swelling and Its Molecular Mechanism", Physiol. Chem. Phys.

- 11 Zhang, Z. L., and Ling, G. N., "Studies on the Physical State of Water in Living Cells and Model Systems. V. Further Studies of the Warming Exothermic Reaction of Frozen Aqueous Solutions of Polyvinylpyrrolidone (Poly (Ethylene Oxide), and Urea-denatured Proteins", Physiol. Chem. Phys.
- 12 Ling, G. N., and Tucker, M., "Only Solid Red Blood Cell Ghosts Transport K^+ and Na^+ Against Concentration Gradients: Hollow Intact Ghosts With K^+ - Na^+ Activated ATPase Do Not", Physiol. Chem. Phys.
- 13 Ling, G. N., and Fisher, A., "Cooperative Interaction Among Cell Surface Sites: Further Evidence in Support of the Surface Adsorption Theory of Cellular Electrical Potential", Physiol. Chem. Phys.
- 14 Ling, G. N., "The Osmotic Activity of Aqueous Solutions of Several Polymers Including Gelatin, Polyvinylpyrrolidone and Poly (Ethylene Oxide) Which Reduce the Solubility of Water for Na^+ , Sugars, and Free Amino Acids", Physiol. Chem. Phys.
- 15 Ling, G. N., Walton, C. L., and Ochsenfeld, M. M., "The Resting Potential of Frog Muscle is Indifferent to External Mg^{++} Even Though Mg^{++} is Substantially More Permeant Than K^+ ", Physiol. Chem. Phys.
- 16 Ling, G. N., "The Physical State of Water and K^+ in Living Cells", 2nd International Conf. on Water and Ions in Biological Systems, National Institute of Health

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EVIDENCE SHOWING THAT THE BULK OF Na^+ IN A SULFONATE ION EXCHANGE RESIN
EXISTS IN AN ADSORBED STATE AND ITS SIGNIFICANCE FOR THE INTERPRETATION OF
NMR DATA ON Na^+ , K^+ AND OTHER IONS IN LIVING CELLS

by

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INTRODUCTION

There are two diametrically opposed views concerning the physical state of the counteranions like Na^+ in a commercially available nuclear sulfonate type of cation exchange resins. In one widely cited view proposed by H. Gregor (1948, 1951) the bulk of the counteranions in the resin exists as free ions. In the opposite view, first presented by Ling in 1952 and later incorporated into the larger theme, the association-induction (AI) hypothesis, the counterions are adsorbed in the classic sense, i.e., one (hydrated) counterion is closely associated with and in direct contact most of the time with one fixed anion of the resin phase. The major force holding the adsorbed ion in close association is electrostatic attraction, enhanced by the phenomenon of dielectric saturation at the near vicinity of a charged ion. (A theory similar to that of Ling was given by Harris and Rice in 1956, apparently unaware of the earlier work.)

The main interest of Ling in 1952 on exchange resins originated from the resemblance between the ability of virtually all living cells to accumulate K^+ selectively over Na^+ and the ability of certain types of cation exchange resins to do the same. The suggestion was then made that in both systems, a similar mechanism operates. Gregor (1948, 1951), on the other hand, had little interest in cell physiology. He suggested an entirely different mechanism based on the assumption of free counteranions in the resin water: as free ions, the smaller, less hydrated K^+ is preferred over the larger, more hydrated Na^+ due to the intense pressure postulated to exist in the exchange resins.

The dichotomy in these two different interpretations of the same phenomenon seen in ion exchange resin produced another set of divergent interpretations of the NMR of ^{23}Na in living cells. Based on the assumption of Na^+ binding in ion exchange resin, Cope (1967) and Ling and Cope (1969) cited the

similar NMR behaviors of ^{23}Na in living cells and in the nuclear sulfonate ion exchange resin, Dowex 50, as evidence for Na^+ adsorption in living cells. Based on the assumption of full counterion dissociation in the same ion exchange resin, the same similarity of NMR behaviors in the two systems led Berendsen and Edzes (1973) to conclude just the opposite, i.e., Na^+ (and K^+) in living cells is all free.

The above are examples of the schism created by the uncertainty of the physical state of the counter cations, in particular Na^+ in an obviously attractive model system, the man-made ion exchange resins. To help resolve this problem, we undertook an investigation of the physical state of Na^+ in solutions of the linear polymers of Na^+ poly-styrene sulfonate, which exists in a solution form, easily amenable to investigation. It is well known that sulfonate ion exchange resins like Dowex 50 are simply these linear polymers (at high concentration) crosslinked into a three dimensional network with the cross-linking agent, divinylbenzene. Since increasing ion concentration decreases the free energy of dissociation and enhances ionic association, the demonstration of a significant degree of counterion association in dilute solutions of the linear polymer would confirm the adsorption concept of the AI hypothesis. On the other hand, the demonstration of complete dissociation of the counter cation would offer support for Gregor's theory.

MATERIALS AND METHODS

The polymers studied were poly-styrene sulfonate (PSS) obtained as several batches of gifts under the commercial name Versa-T1[®] 400 and Versa-T1[®] 500 from Proctor Chemical Company, a subsidiary of National Starch and Chemical Corp., Bridgewater, NJ. The approximate molecular weight of the Versa-T1[®] 500 is 500,000 daltons; that of Versa-T1[®] 400, 400,000 daltons.

All the chemicals used were of the reagent grade. Guanidine HCl and choline chloride were from Eastman, Rochester, N. Y. Arginine HCl and lysine HCl were from Sigma Chemical Co., St. Louis, Mo.

The Na electrode (Corning 476-210) used in the early part of this work was a gift from Corning Glass Works, Medfield, MA, when this electrode was still not in production. However, this fine electrode is commercially available now. Made with glass of NAS 11-18 composition, this electrode has high specificity for Na^+ . Thus at pH 7, the Na^+/K^+ selectivity is about 1000; that of Na^+/Li^+ , 250. For maximum reproducibility we kept the electrode bulbs immersed in a 0.1 N NaCl at all times when not in use. The reference electrode used was a single junction calomel electrode (Model 90-01, Orion Res. Inc.) connected to the measuring vessel by a salt bridge of heavy wall capillary tubing filled with 0.1 N KCl and 2% agar. The end of this salt bridge in the solution to be measured is drawn to a very small diameter with the tip inner bore of less than 1 mm. The electrodes were coupled to either a Fisher pH meter Model 620, or a Beckman pH meter Model 4500. The output of the meter was fed into a Linseis multi-channel strip-chart recorder and readings were taken after steady levels were reached. This usually took 2 to 3 minutes. As a rule new standard curves were constructed each day. However, reading of an unknown sample was always sandwiched between a pair of readings of standards above and below the reading of the unknown sample.

For the determination of total Na^+ concentration of the polymer solutions, we relied on atomic adsorption spectrophotometry (Perkin-Elmer Model 103). Extreme care was exercised in diluting the viscous samples to assure complete homogeneity. The samples diluted to contain Na^+ concentration in the range, 5 to 100 μM , were read in the presence of a constant concentration of LiCl (97 mM) and $\text{NH}_4\text{H}_2\text{PO}_4$ (3.0 mM) which served as radiation buffers.

RESULT

Determination of Free and Bound Na⁺

We chose two methods to convert the polystyrene sulfonate (PSS) into the Na⁺ form: (i) extensive dialysis with repeated changes of solutions of 1 M NaCl and (ii) by conversion of PSS first into a H⁺ form after overnight exposure of PSS in a dialysis bag to 1 N HCl at 4^o C and by subsequent dialysis against repeated changes of 1 M NaCl until neutrality was reached. The results are similar; both methods produced pure Na⁺ polymers. PSS in the Na⁺ form thus obtained is then further dialyzed against distilled water adjusted to pH 11 with NaOH. The dry weight of aliquots of the stock solution was determined by heating in vacuo at 100^o C. Some aliquots were diluted with similar basic distilled water to the various desired concentrations before their free Na⁺ concentrations were measured with the Corning 476-210 Na electrode. Other aliquots were diluted to the proper concentration range for the determination of total Na⁺ with the aid of atomic absorption spectrometry.

Figure 1 shows the result of a series of such measurements. From the total Na⁺ concentration one estimates a Na⁺ concentration of 5 mmoles of Na⁺ per gram of dry NaPSS, which is slightly higher than that predicted on the assumption of a uniform monomer formula $(-\text{CH}_2-\overset{\text{H}}{\underset{\text{SO}_3^-}{\text{C}}}-)_n$, which has a monomer weight

of 206.20 and a predicted Na⁺ content of 4.85 mmoles/g. NaPSS.

The free Na⁺ concentration for each of these samples as indicated in Fig. 1 fell far below the level of total Na⁺ concentrations measured. By subtraction, one obtains the bound fraction of Na⁺. Figure 2 combines the results of data given in Fig. 1 and another set of data. The ordinate represents adsorbed Na⁺ expressed as a percentage of total Na⁺. To be noted is that

the percentage of adsorbed Na^+ was low in very dilute solutions of NaPSS but it increased sharply with increase of NaPSS concentration until at about 4% NaPSS, the adsorbed Na^+ makes up 80% of the total Na^+ . From 4% on, the adsorbed Na^+ rose much less steeply to still higher levels.

Demonstration of Specificity in Cation Adsorption

The results presented in Figures 1 and 2 show that the bulk of Na^+ in solutions of NaPSS is not "seen" by the Na^+ electrode. The simplest interpretation is that the bulk of Na^+ is adsorbed. However, there is also the theoretical possibility that the "invisible" Na^+ reflects not a one Na^+ -one sulfonate anionic site close contact adsorption, but rather fully dissociated Na^+ hovering around in the vicinity of the anionic sites in spaces not accessible to the electrode.

There is a method to distinguish between these two alternative conditions of the "invisible" Na^+ (Ling and Ochsenfeld, 1966; Ling, 1977). This method relies on the employment of two or more competing ions which, like Na^+ , carry in each case a net single positive charge and are thus quite alike in their long-range attributes but differ from one another in short-range attributes. If the "invisible" Na^+ is not in direct contact with the anionic site, then the displacement effect of a pair of these competing cations would depend only on their identical long-range attributes (i.e., their valency) and are thus indistinguishable. If on the other hand, the "invisible" Na^+ is

in close contact with the anionic sites, then the degree of displacement by two such ions with different short-range attributes may be quite different.

As competing ions, we chose the chloride salts of four "cations" that carry a single net positive charge: arginine HCl, guanidine HCl, choline chloride, and lysine HCl.

Before testing their effects on displacing Na^+ from PSS, we must first establish that these cations do not by themselves significantly alter the accuracy of the Na^+ electrode to register (only) the free Na^+ concentration in a solution. To do so, we determined the Na^+ concentration at the concentrations of 10^{-5} , 10^{-4} , and 10^{-3} M in the absence and presence of each of these competing ions at concentrations 100 times higher than the respective Na^+ concentration. The results (Table 1) show that the deviation in the electrode reading is less than 2%. Since in the actual experimental measurements to be described, the competing ion concentrations were not 100 times higher than the Na^+ concentration but were at most only .5 times higher than that of the Na^+ concentration, there was in essence no direct significant interference of these ions on the Na^+ electrode and that the Na^+ electrode continued to faithfully monitor only the free Na^+ concentration present.

Figure 3 presents a typical experiment in which the effects of varying the concentration of the competing ions (indicated on the abscissa) on the percentage of displaced bound Na^+ . Note that the bulk of bound Na^+ (i.e., 77%) are displaced by 300 mM guanidine. However, at 100 mM concentration, the most effective displacement was not guanidine but arginine. (Due to its lower solubility, we could not study the effect of arginine at the highest concentrations as we did in the case of the 3 other cations.) The great difference between the effectiveness of arginine and that of lysine, both trifunctional amino acids, clearly shows that the ineffectiveness of lysine in displacing bound Na^+ was not due to its possession of the additional α -amino and α -carbonyl groups, a feature shared by both lysine and arginine. The data presented in Fig. 3 shows that there was a high degree of specificity in the effectiveness of displacing the Na^+ even though all carry a single net positive electric charge and thus would have, identical effectiveness if they had acted only

to displace the "invisible" Na^+ through their long-range attributes. In addition, the data show that the short-range attributes played major roles in the displacement of the Na^+ , thereby proving that the "invisible" Na^+ is indeed adsorbed in the sense that the Na^+ is in close contact with the anionic sites in a one Na^+ -one sulfonate group relationship.

Since guanidine can displace 77% of the bound Na^+ while lysine at equal concentration can only replace 8% of the bound Na^+ , at least $77 - 8\% = 69\%$ of the "invisible" Na^+ is in the close-contact adsorbed state. However, the slope of the guanidine curve at 300 mM is considerably higher than that of the lysine curve. Thus one may safely conclude that more than 69% of the "invisible" Na^+ is in the direct-contact adsorbed state in the PSS solution.

DISCUSSION

The results of our investigation presented above left no doubt that the bulk of Na^+ in a solution of Na PSS at concentrations higher than 2% is in a close-contact adsorbed state. As a rule, with the increase of the concentration of Na PSS, the fraction of Na^+ existing in this adsorbed state increases. Commercial nuclear sulfonic acid ion exchange resins like Dowex 50 are cross-linked polystyrene sulfonate just like the Versa-T1 we studied. The total solid content of Dowex 50 and other similar resins are in the 40% to 50% range and are higher than the highest concentration of Na PSS we studied (i.e., 35%, see Fig. 1). Therefore we have little doubt that virtually all Na^+ in a Dowex-50 exists in a close-contact adsorbed form.

These findings therefore disprove Gregor's theory of total counterion dissociation in sulfonate ion exchange resin and clearly confirms the prediction of the AI hypothesis.

As mentioned earlier, the present finding that the counterions in Dowex 50 as well as other types of ion exchange resins are in an adsorbed state also offers simple explanations for much of the characteristics of ion exchange resin properties that Gregor's theory could not explain, including the much higher preference for Ag^+ , Tl^+ over Cs^+ and other monovalent cations of equal size (Helfferich, 1962) and the diametrically opposite selective preference of Na^+ vs. K^+ when the functional groups differ: preference for K^+ over Na^+ in sulfonate resins; preference for Na^+ over K^+ in phosphoric and carboxylic resins (Bregman, 1953). The model presented by the AI hypothesis also offers an explanation why in resin bearing the same functional groups (nuclear sulfonate), an increase of the percentage of cross-linking agent DVB, causes a "selectivity reversal" from preferring K^+ over Na^+ and one favoring Na^+ over K^+ (Reichenberg, 1951, 1955; Bregman, 1953), a finding that also cannot be explained by Gregor's theory (see Helfferich, 1962, p. 159) but agree well with the theoretically calculated relations between the electron density at the acidic group (i.e., the c-value) and that incorporation of DVB changes this electric density (see Reichenberg, 1966; also Ling, 1981).

The conclusion derived from the present studies also reaffirms the parallelism seen thirty years ago between the mechanism of selective K^+ accumulation in ion exchange resin and in living cells (Ling, 1952, 1962, 1983), since it is now firmly established that K^+ in living muscle cells also exists in an adsorbed state, by three laboratories, using a total of four independent methods: autoradiography (Ling, 1977, Edelmann, 1980); transmission electron microscopy (Edelmann, 1977); dispersive x-ray microprobe analysis (Edelmann, 1978; Trombitas and Tigyi-Sebes, 1979); and laser mass spectrometer microprobe analysis (LAMMA) (Edelmann, 1981).

In the fifties, Jardetsky (1956) and Jardetsky and Wertz (1956a, b) studied Na^+ complexing in various solutions and other systems including the sulfonate ion exchange resins, Dowex 50. They found that only about half of the Na^+ in Dowex 50 resin was NMR-visible. They suggested that there are two fractions of Na^+ in the resin: one free and is NMR visible, the other adsorbed and is NMR invisible. Accepting this logic, Cope (1967) studied Na^+ NMR of living tissues, arriving at the conclusion that much of the Na^+ in living tissues exists in an adsorbed state. Later Ling and Cope (1969) measured the Na^+ NMR of frog muscle whose K^+ has been replaced stoichiometrically by Na^+ . They also used the same assumption of Jardetsky and Wertz, and concluded that muscle K^+ is in an adsorbed state. Many other studies of a similar type rapidly followed (see Ling, 1984) until in 1972 this trend was abruptly brought to a stop by Berendsen and Edzes (1973).

Berendsen and Edzes (1973) pointed out that the disappearance of part of the Na^+ signal is not due to partial binding. Instead, according to them, it reflects a 40-60 splitting of the Na^+ signal when the Na^+ nuclei (which has a quadruple moment) is in an electric field gradient. They then postulated that the electric field gradient in living cells to be a diffuse one, spread over domains of 100 \AA or wider. To support their view, they showed that in deteriorated muscle (deteriorated to such an extent that "it swelled") and in Dowex 50 ion exchange resin charged with Na^+ , the Na^+ signals show similar 40-60% behaviors as well as other characteristics. They based their argument on the assumption that all Na^+ in Dowex 50 resin exists in a free-form, much as the widely known theory of Gregor had predicted. They did not explain how can a diffuse gradient over a space of 100 \AA be generated in Dowex 50. Thus with a total anionic site density of 2 M in these resins the average charge-to-charge distance should be closer to $(2 \times 6.06 \times 10^{23})^{1/3}$ or 1.06 \AA rather than 100 \AA .

The present proof that the bulk of Na^+ in Dowex 50 is adsorbed shows that the evidence Berendsen and Edzes used to support the diffuse electric field gradient idea is wrong: counteraction Na^+ is in close contact with fixed sulfonate groups. The similarity they demonstrated in the NMR behavior of deteriorating muscle and Dowex 50, only established that Na^+ in the deteriorating muscle also existed in an adsorbed state. Whether this Na^+ was adsorbed on the proteins of the dead muscles or perhaps even in the bacteria growing in the sample that had already produced the bad smell, remains to be determined.

It is also appropriate to point out that in 1978 Chang and Woessner (1978) from purely theoretical grounds reached the conclusion that the electric field gradient required to produce the NMR behavior seen must be a great deal steeper than Berendsen and Edzes envisaged. In fact, they argued that a reasonable estimate gives a gradient over such a short distance that it is quite compatible with that of a Na^+ adsorbed on a negatively charged site. Ironically, Edzes, Ginzburg, Ginzburg, and Berendsen (1977, p. 733) themselves seemed to have changed their early view and had suggested that the 40-60 split of ^{23}Na and other nuclei in an electric field gradient can be the result of "binding of an ion in a specific site." But this was not new either. Lindblom (1971) long ago come to a similar conclusion.

These more recent development have fully restored our confidence that NMR study of Na^+ and K^+ was and still is an exciting and highly useful approach to understanding the cell physiology of ions and that past conclusions concerning the adsorbed state of Na^+ in living cells and model systems of Cope and others are qualitatively quite correct even though quantitatively, the amount of adsorbed Na^+ might have been underestimated (see Ling, 1984).

SUMMARY

Using Na^+ specific glass electrode, the free Na^+ concentrations in solutions of Na polystyrene sulfonate (Na PSS) were measured. The data showed at Na PSS concentration equal to or higher than 5%, 80% or more of the Na^+ exists in an adsorbed state in the sense that Na^+ exists in close contact with the sulfonate group, one Na^+ to one anionic site and that the degree of association increased with PSS concentration. Since Dowex 50 ion exchange resins are cross-linked PSS at high concentration (40% to 50%) the present finding showed that ^{23}Na NMR can indeed determine the adsorbed Na^+ in living cells as was once believed though in a quantitatively incorrect way but erroneously challenged.

ACKNOWLEDGMENT

We are most indebted in general to the National Starch and Chemical Company for their generous gifts of Versa-T1 and other highly valuable experimental samples and in particular to Ms. Patricia Gleason for her

helpfulness. We also thank Corning Glass Works for their generous gift of their then as yet unmarketed Na^+ sensitive glass electrode. Mary A. Brady for her earlier work on the subject and Jean Brogan for her able help in preparing the manuscript.

This investigation was supported by Office of Naval Research Contract N00014-79-0126; NIH Grants 2-R01-CA16301-3 and 2-R01-GM11422-13; and Pennsylvania Hospital General Research Support.

LEGENDS

- Figure 1 - The concentration of total Na^+ and free Na^+ in solutions of Na^+ polystyrene sulfonate (Na^+PS). The abscissa represents the concentration of NaPS expressed as percentage (W/V). The ordinate represents the concentration of total Na^+ and free Na^+ both in molarity. The free Na^+ represents that which was detected by the Na^+ sensitive electrode.
- Figure 2 - The fraction of Na^+ that exists in a bound form not detected by the Na^+ electrode in different concentrations of Na^+ polystyrene sulfonate.
- Figure 3 - Fractional displacement of bound Na^+ in solutions of Na PSS (5%)
The ordinate represents the percentage of bound Na^+ that has been displaced by the competing ions. The abscissa represents the concentration of the competing ions.
- Table 1 - Effect of competing ions on the Na^+ electrode readings in millivolts in solution containing 10^{-5} , 10^{-4} , and 10^{-3} M free Na^+ . Competing ions were at concentrations 100 times higher than the concentration of Na^+ present.

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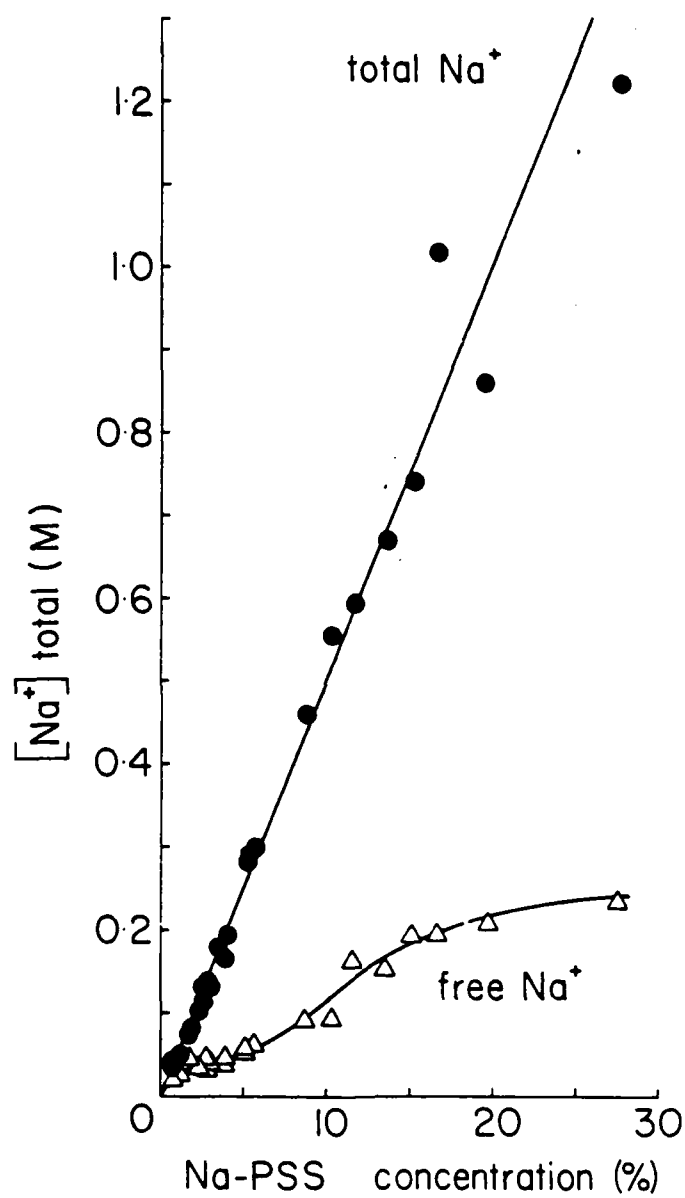


FIGURE 1

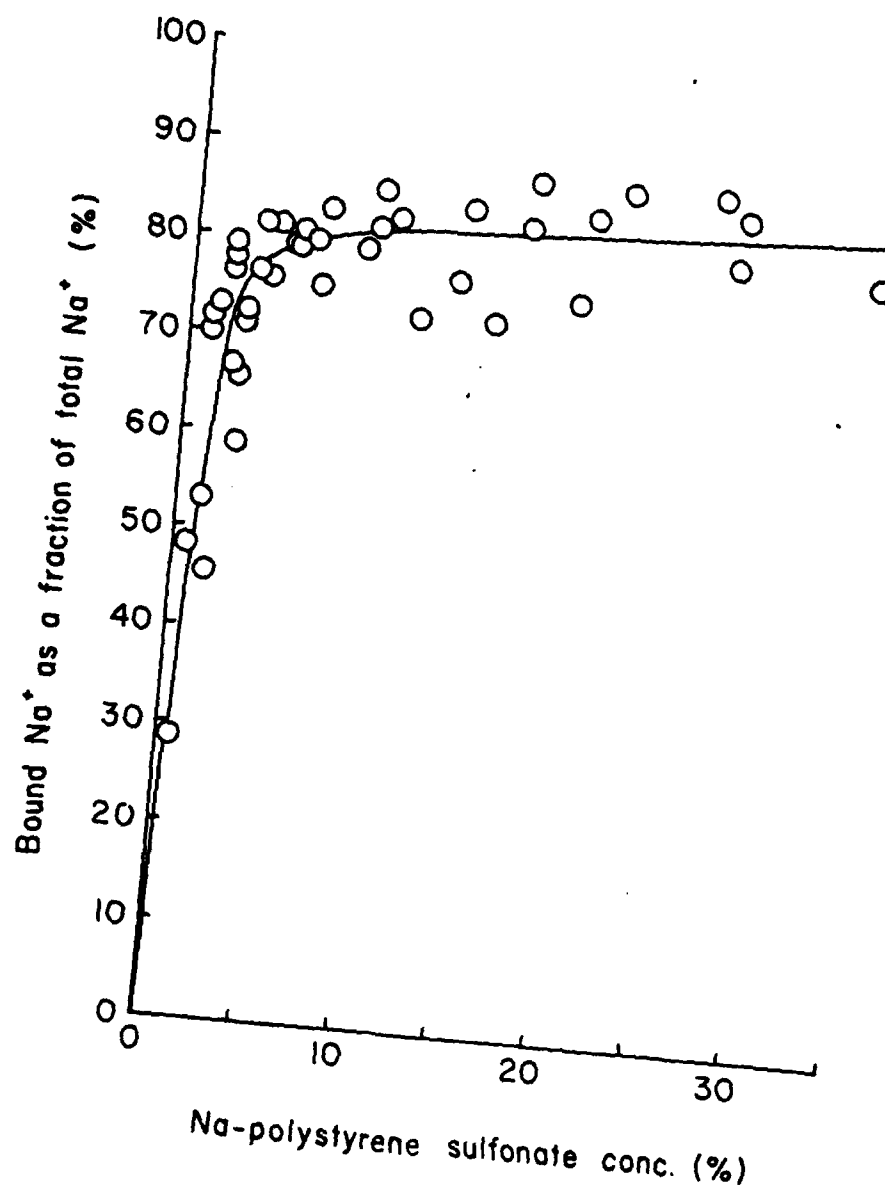


FIGURE 2

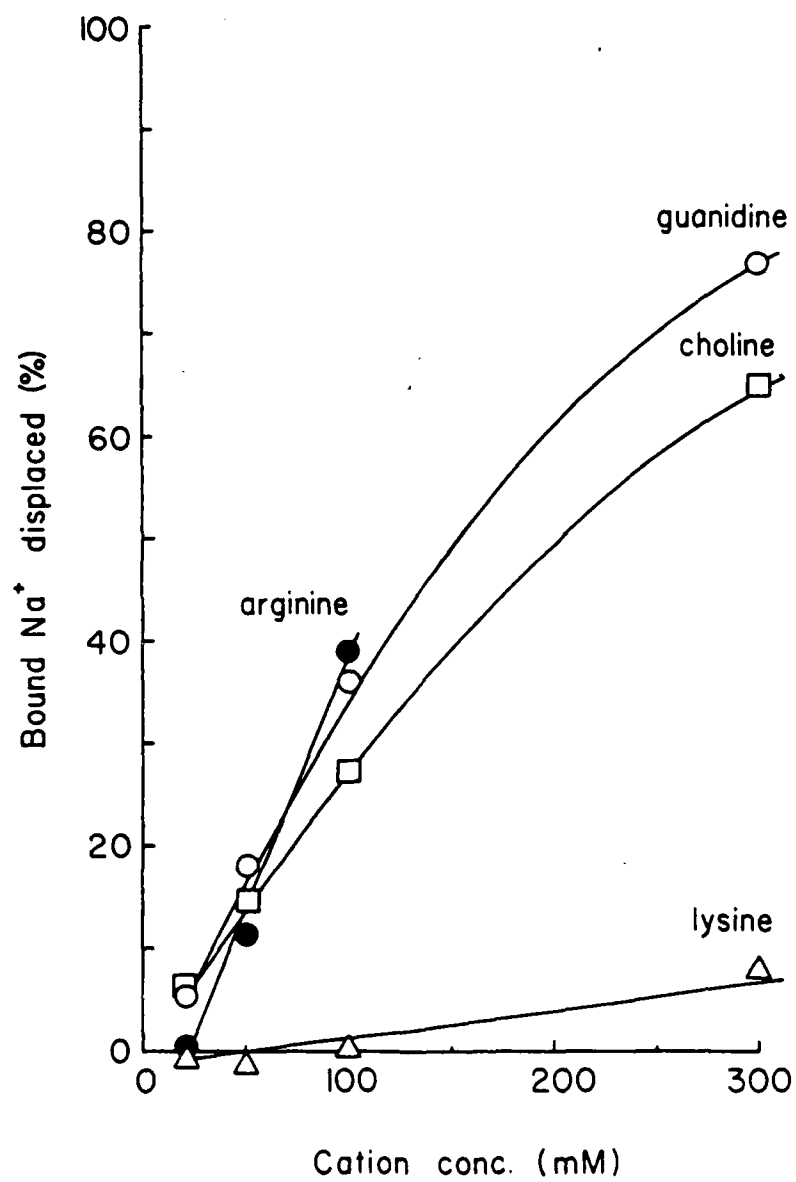


FIGURE 3

Competing Ion (100 X C _{Na})(M)	Concentration of Na ⁺ (C _{Na}) (M)		
	<u>10⁻³</u>	<u>10⁻⁴</u>	<u>10⁻⁵</u>
None	-81.6	-140.3	-195.1
Arginine	-81.9	-139.7	-192.8
Choline	-83.4	-140.3	-195.2
Guanidine	-83.7	-141.3	-196.1
Lysine	-82.5	-141.5	-203.5

TABLE 1

The Association-induction hypothesis
A theoretical Foundation Provided by possible beneficial Effects
of a low Na, high K Diet and other similar Regimens
in the Treatment of Patients suffering from debilitating illnesses

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*Éléments d'une théorie explicative des effets bénéfiques du régime pauvre en Na et riche en K
et de ses analogues, dans le traitement de sujets souffrant de maladies débilitantes.*

Après un bref rappel de l'histoire de la physiologie cellulaire cet article résume quelques arguments qui rendent aujourd'hui l'hypothèse conventionnelle, largement enseignée, de la pompe membranaire insoutenable. Une autre théorie, l'hypothèse association-induction (AI hypothesis) est présentée avec les éléments théoriques qu'elle apporte pour comprendre les effets bénéfiques d'un régime pauvre en Na et riche en K, ou autres comparables, pour le traitement de sujets souffrant d'une maladie débilitante.

In 1805, thirty years before Schleiden and Schwann's pronouncement of the Cell Theory, Lorenz Oken, in his monograph "Die Zeugung" (The Creation), clearly described living cells as the basis of life. The living cell in turn, according to Oken, is made of "Urschleim", which later was named "protoplasm".

Some sixty years later Moritz Traube (1867) discovered an artificial membrane made by mixing copper sulfate with K ferrocyanide, which like living membranes was semipermeable, i.e., permeable to water but not to ions, sugars, and other solutes. Using the copper ferrocyanide membrane as a model, plant physiologist Wilhelm Pfeffer (1877) proposed the membrane theory which was to dominate the thinking of biologists for fully a whole century to come. Traube offered an "atomic sieve" theory to explain the semipermeability of the copper ferrocyanide membrane. That is the membrane has pores just big enough to allow the passage of water molecules but not the larger sugar and ions, etc. Later electron and x-ray diffraction studies disproved Traube's theory. The interstices (100 to 200 Å) are much bigger than solutes to which it is impermeable (e.g., sucrose, 9.4 Å) (see Glassstone, 1946).

A contemporary of Traube, English chemist Thomas Graham introduced colloidal chemistry. Graham (1861) stated "As gelatine appears to be its type, it is proposed to designate substance of the class as colloids (Kolloda, glue) and to speak of their peculiar form of aggregate as colloidal condition of matter". Graham noted two characteristics of the colloids: slow diffusibility and a gelatinous consistency when hydrated. Among the colloids that Graham discussed was copper ferrocyanide gel, the substance that is nearly perfectly semipermeable when cast in the form of a membrane as Traube did.

The recognition of cells as the basic unit of life implies that living matter is not a continuous mass but consists of separate units. This discontinuity between cell and cell as well as the discontinuity between cell and its aqueous environment is selective in a subtle manner. Thus from the earliest days of biology, it was recognized that water can move in and out of cells with relative ease. The near-perfect semipermeable properties of copper ferrocyanide gel membrane was thus at one time considered to be duplicating the properties of all living cells.

Perhaps the failure of the atomic sieve idea, first suggested by Traube and later revived by Boyle and Conway (1941) might be partially responsible for the increasing acceptance of the lipoidal membrane theory of Overton (1896). According to this view, the cell membrane is not colloidal in nature as Traube's copper ferrocyanide gel is, but is a lipid sharing basic properties with olive oil, for example. Solubility in the lipid membrane in this hypothesis determines the relative permeability of a particular solute.

In years following, especially after the advent of radioactive tracer technique, it became clear that the living cell membrane is not just permeable to water but is also permeable to a host of other solutes dissolved in water. The most surprising of this new revelation concerns the permeability of sugar, free amino acids, and Na^+ (salt), which at high strength cause sustained cell shrinkage and had therefore been long considered as impermeant. The discovered permeability of these solutes posed other questions.

If the cell membrane is permeable to a particular solute, one expects that over a long period of time, this solute would reach and be maintained in the cell water at the same concentration as that in the external medium. Yet old cells as well as young cells share the striking characteristic of maintaining the same high level of K^+ and the same low level of Na^+ in the cell water while the aqueous environment in which these cells are bathed contain as a rule a low level of K^+ and a high level of Na^+ (Figure 1).

To meet the new challenge, the Na pump hypothesis was suggested and in due time accepted by many scientists. In this hypothesis the low level of Na^+ was achieved by certain devices in the lipid cell membrane called pumps. These Na pumps continually extrude Na^+ from the cell so that a low level in the cell of this ion is maintained in spite of its constant inward diffusion into the cells (Dean, 1941).

At first glance, the Na pump concept seems reasonable enough. There is no question that Na^+ can be transported against concentration gradient as across certain specialized tissues such as frog skin and intestinal epithelium. The question that must be answered is, "Is there enough energy to operate the pumps for all living cells?"

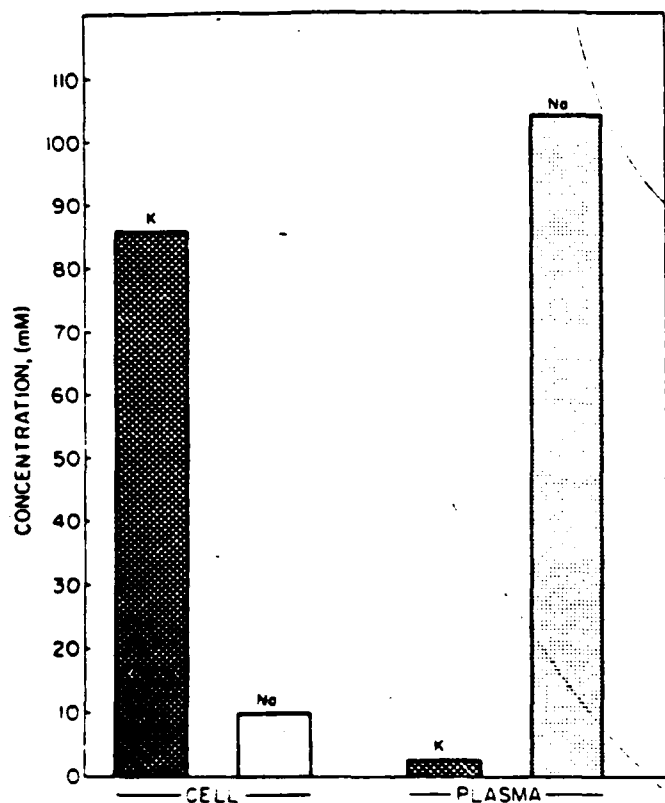


Figure 1 - The asymmetrical distribution of K⁺ and Na⁺ between living cells (frog muscle) and its environment, the plasma.

To move a mole of positively charged Na⁺ from the cell inside to the outside requires the expenditure of energy, in order to overcome the unfavorable electrochemical potential gradient due to the higher concentration of Na⁺ in the outside medium than the cell and the cellular resting potential which is positive on the outside. The polarity and magnitude of the electrochemical gradient also dictate that virtually all the outward Na⁺ movement has to be due to active pumping if the basic assumption of free K⁺ and free water in the cell are taken as correct (see below). By labelling intracellular Na⁺ with radioactive tracer, the minimal energy need of the Na pump could be calculated from the efflux rate, the resting potential and the intra-extracellular Na⁺ concentration ratio. While early work suggests that the Na pump would consume only a not unreasonable amount of energy (Levi and Ussing, 1948) a more stringently controlled study led to an altogether different conclusion: the Na pump, alone would consume from 15 to 30 times as much energy as the cell commands (Ling, 1962).

Both the basic finding of Ling that the efflux rate Na⁺ from frog muscles was not affected by the arrest of both aerobic and glycolytic metabolism (Keynes and Maisel, 1945; Conway, Kernan and Zadumarsky, 1961) and the findings of excessive energy need for the pumps (Jones, 1965; Minkoff and Damadian, 1973) were repeatedly confirmed. Three remedial hypothesis: exchange diffusion, Na⁺ sequestration in open sarcoplasm reticulum and nonenergy consuming Na pumps were each experimentally disproven (for review, see Ling, Walton and Ling, 1979).

If we take on the realistic task for the cell provide energy to operate not just a Na pump but a long and steadily lengthen-

ing list of other pumps beside the Na pump, the finding of excessive energy need of the Na pump alone, in my belief, is enough to disprove the pump hypothesis. Apparently only a few scientists shared this view with me at that time. However, as time went by, other new evidence have been accumulating also against the membrane-pump concept. Several of these will be briefly mentioned: (1) Squid axon membrane sheath with the axoplasm removed remained anatomically as well as functionally intact. Yet it does not pump Na⁺ outward and K⁺ inward in the presence of ATP and thus recreate the K⁺ and Na⁺ distribution pattern seen in normal squid axons as the membrane pump hypothesis predicts (Ling, 1965; Ling and Negendank, 1980); (2) Frog muscle cells whose cell membrane (and postulated pumps) is made non-functional by being partially amputated and partially suspended in air and thus deprived of "sinks" or "sources" for the pumps can nevertheless accumulate K⁺ and exclude Na⁺ in its cytoplasm much as intact cells do (Ling, 1978); (3) There is gathering evidence that lipids in the cell membrane do not form a continuous layer but exist only in pocket or "islands". Anatomical evidence for this view was provided by the indifference of the trilaminar structure of unit membranes to removal of 95 % of membrane lipids prior to fixation (Fleischer and Stoeckenius, 1967; Morowitz and Terry, 1969); by careful procedures to prevent membrane protein denaturation (Sjöstrand and Bernhard, 1976) and by the failure of specific K⁺ ionophores, valinomycin and monactin to increase cell membrane permeability to K⁺ while a model membrane of continuous layer of natural or synthetic phospholipids increases its K⁺ permeability by several orders of magnitude on response to these agents (Stillman, Gilbert and Robbin, 1970; Maloff, Scordilis et al., 1978; Ling and Ochsenfeld, 1978; Jain, 1972); (4) Unanimous evidence from three different laboratories across the world using at least three different methods that the bulk of intracellular K⁺ is in an adsorbed state, thereby directly contradicting one of the basic tenets of the membrane theory, i.e., the major intracellular K⁺ as well as the bulk of intracellular water exists in the free state. This subject will be taken up in more detail again.

This powerful array of diverse and mutually supportive evidence contradicting the membrane-pump theory, made it mandatory to seek a new framework of thinking, a new paradigm of the living cell such as that provided by the association-induction hypothesis (AI Hypothesis).

The Physical State of Ions, and Water in the Resting Living Cells According to the Association-Induction Hypothesis.

Contrary to the membrane pump theory, the major cation in the cell, K⁺ as well as the entire amount of water (excluding that in vacuoles, etc.) in a resting normal living cell is in an adsorbed state. K⁺ is preferentially (over Na⁺) adsorbed on β- and γ-carboxyl groups of certain cellular proteins while water is adsorbed in polarized multilayers on a matrix of extended protein chains (Ling, 1952, 1965 a, b, 1969, 1977 a, Ling, Miller and Ochsenfeld, 1973) (Figure 2).

Thus the reason K⁺ is accumulated to a higher level in the cell than in the external environment is due to the preference of the β- and γ-carboxyl groups (under the condition of the living, resting cells) for K⁺ over, say Na⁺. The low level of Na⁺ in the cell is largely due to the reduced solubility of water existing in the state of polarized multilayers for (hydrated) Na⁺ as well as K⁺, sugars, and amino acids. However, the cell proteins serving these functions do not do so always; they do so only when they are found in the proper "environment", conducive to the maintenance of the protein-ion-water assembly in a resting high energy living state to be described next. This "environment" includes the availability of ATP.

TABLE I

Temperature was $25 \pm 1^\circ\text{C}$ and test tubes were agitated, except in the experiments of E, which were carried out at $0 \pm 1^\circ\text{C}$ and in which some test tubes, marked Q, were quiescent and unstirred. S represents sacs shaken in test tubes at 30 excursions/min (each excursion spans 1 inch) except the first set (S') for which agitation was achieved by to-and-fro movement of silicone-rubber coated lead shot within the sacs. The symbols *a* and *b* indicate that the media contained initially 1.5 M Na_2SO_4 and 0.5 M Na-citrate respectively. In D, poly(ethylene oxide) (mol.wt. 600,000) was dissolved as a 10% (w/w) solution, and the viscous solution was vigorously stirred before being introduced into dialysis tubing. In E, the quiescent samples contained more water. This higher water content accounts for only a minor part of the difference, as shown by comparison of the 6th and 7th sets of data: even with a larger water content, the ρ -value is lower in the stirred samples (6th). Na was labeled with ^{22}Na and assayed with a γ -counter. (By permission of *Physiol. Chem. Phys.*).

29 Symbols

Group	Polymer	Concentration of medium (M)	Number of assays	Water content (%) (mean \pm SE)	ρ -Value (mean \pm SE)
(A)	Albumin (bovine serum)	1.5 <i>a</i>	4	81.9 ± 0.063	0.973 ± 0.005
	Albumin (egg)	1.5 <i>a</i>	4	82.1 ± 0.058	1.000 ± 0.016
	Chondroitin sulfate	1.5 <i>a</i>	4	84.2 ± 0.061	1.009 ± 0.003
	α -Chymotrypsinogen	1.5 <i>a</i>	4	82.7 ± 0.089	1.004 ± 0.009
	Fibrinogen	1.5 <i>a</i>	4	82.8 ± 0.12	1.004 ± 0.002
	γ -Globulin (bovine)	1.5 <i>a</i>	4	82.0 ± 0.16	1.004 ± 0.004
	γ -Globulin (human)	1.5 <i>a</i>	4	83.5 ± 0.16	1.016 ± 0.005
	Hemoglobin	1.5 <i>a</i>	4	73.7 ± 0.073	0.923 ± 0.006
	β -Lactoglobulin	1.5 <i>a</i>	4	82.6 ± 0.029	0.991 ± 0.005
	Lysozyme	1.5 <i>a</i>	4	82.0 ± 0.085	1.009 ± 0.005
	Pepsin	1.5 <i>a</i>	4	83.4 ± 0.11	1.031 ± 0.006
	Protamine	1.5 <i>a</i>	4	83.9 ± 0.10	0.990 ± 0.020
	Ribonuclease	1.5 <i>a</i>	4	79.9 ± 0.19	0.984 ± 0.006
(B)	Gelatin	1.5 <i>a</i>	37	57.0 ± 1.1	0.537 ± 0.013
(C)	PVP	1.5 <i>a</i>	8	61.0 ± 0.30	0.239 ± 0.005
(D)	Poly(ethylene oxide)	0.75 <i>a</i>	5	81.1 ± 0.34	0.475 ± 0.009
		0.5 <i>a</i>	5	89.2 ± 0.06	0.623 ± 0.011
		0.1 <i>a</i>	5	91.1 ± 0.162	0.754 ± 0.015
(E)	PVP Q	0.2 <i>b</i>	4	89.9 ± 0.06	0.955 ± 0.004
	S'	0.2 <i>b</i>	4	87.2 ± 0.05	0.865 ± 0.004
	Q	0.5 <i>b</i>	3	83.3 ± 0.09	0.768 ± 0.012
	S	0.5 <i>b</i>	3	81.8 ± 0.07	0.685 ± 0.007
	Q	1.0 <i>b</i>	3	67.0 ± 0.26	0.448 ± 0.012
	S	1.0 <i>b</i>	3	66.6 ± 0.006	0.294 ± 0.008
	Q	1.5 <i>b</i>	3	56.3 ± 0.87	0.313 ± 0.025
	S	1.5 <i>b</i>	3	55.0 ± 1.00	0.220 ± 0.021

ρ -Values of Na^+ in Water Containing Native Proteins (A), Gelatin (B), PVP (C,E), and Poly(Ethylene Oxide) (D).

The Living State and ATP

The physical state of liquid water is different from that of solid ice not because individual water molecules are different but rather because the relationships among these individual molecules in a space-time coordinate are different. According to the AI Hypothesis, being alive signifies not only just the presence of the right composition of chemical compounds but that they must maintain a special relation to one another both sterically and electronically. The unique state is called the living state: it is a high energy state in the same sense that a cocked gun, a bent bow, and a set mousetrap are in a high energy state.

The maintenance of the high energy living state of the protein-ion-water system depends on interaction of the protein with

certain key compounds called *cardinal adsorbents* (Ling, 1962). One of the most important cardinal adsorbents is ATP. This main product of metabolism, serves to energize biological work performance by electronic polarization of the protein molecules (Ling, 1977 a). The hydrolysis of ATP by the ATPase provides an effective and prompt means of removing ATP, thereby tipping the protein-ion-water system into a lower energy active state as exemplified by a contracted muscle. Energy is injected into the system by ATP resynthesis and resorption onto the cardinal site. A diagram which illustrates the interaction of ATP, protein, ion and water is shown in Figure 3. Here interaction of ATP with the protein's molecules maintains the protein in such an electronic and steric conformation that K^+ is preferentially adsorbed on the β - and γ -carboxyl chains and the backbone NHCO group react with and polarize multilayers of water.

5d. ...

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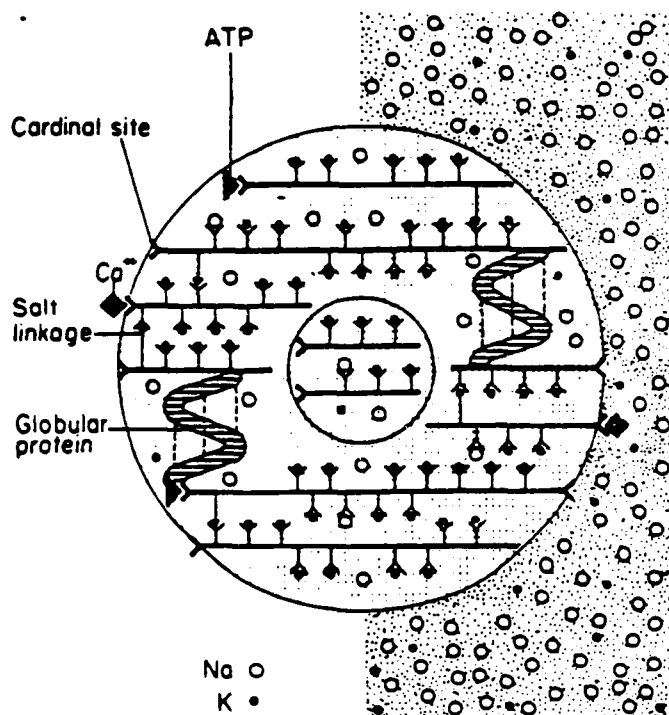


Figure 2 - Diagrammatic illustration of a living cell. Regularly-arrayed dots in the cells represent water molecules existing in the state of polarized layers. Random dots outside the cell represent normal liquid water. Empty circles represent Na^+ ; solid one, K^+ .

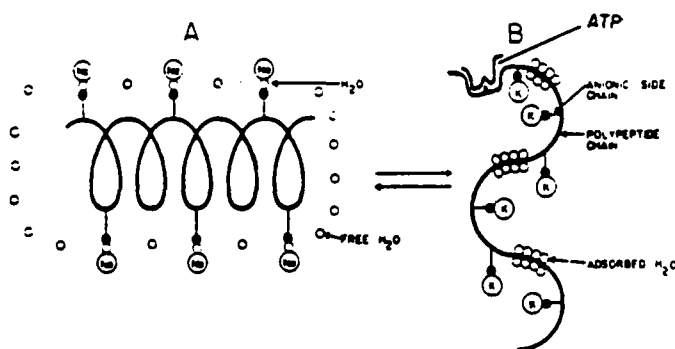


Figure 3 - Diagram of a portion of a protein molecule undergoing auto-cooperative transformation. For simplicity, adsorbed water molecules in multilayers are shown as a single layer. w-shaped symbol represents a cardinal adsorbent, from Ling (1969) by permission of Inter. Rev. Cytol.

Evidence for the Adsorbed State of K^+ in Frog Muscle Cells

Since in voluntary muscle more than 60 % of the β - and γ -carboxyl groups are carried by myosin and since myosin is exclusively found in the A band of the myofibrils, the idea that β - and γ -carboxyl groups adsorb K^+ demands that the bulk of K^+ be found in the A band (Ling, 1977 b). Furthermore, since β - and γ -carboxyl groups are also known to be the sites for uranium binding in conventional electron microscopic sections of tissue proteins, the theory also demands that K^+ in striated

muscle cells be located at the same subcellular structure that stains dark in EM pictures. Figure 4 A shows the stain of uranium marked the concentration of β - and γ -carboxyl groups on the edges of the A bands and the Z-line which bisects the I band.



Figure 4 - Electron micrographs of dry cut, unstained section of freeze-dried frog sartorius muscle. A, Muscle fixed and stained with uranium-lead by conventional procedure. B, EM of Cs^+ -loaded muscle without chemical fixation or staining. C, Same as B after exposure of section to moist air, which causes the hitherto even distribution of thallium to form granular deposits in the A band. D, Section of central portion of B after loading in distilled water. E, Normal " K^+ -loaded" muscle. (A, Partial reproduction of EM from Huxley (1953)) B to F, from Edelmann (1977) (By permission of Physiol. Chem. Phys.).

To test these expectations, the first obstacle to overcome was to prevent K^+ from diffusing away from its natural sites of residence during the preparation of the electron microscope section. This was accomplished by Edelmann (1978 a) with a simple and very effective freeze-drying method he developed. The second step was to replace the light K^+ atom by more dense ions so that they can be visible in the electron microscope. Cs and Tl ions are electron dense; techniques had been previously perfected for the replacement of K^+ by these ions in surviving frog muscle (Ling and Ochsenfeld, 1966; Ling, 1977 c). When these Cs^+ or Tl^+ loaded muscles were frozen dried, imbedded and sections dry-cut, pictures like those shown in Figure 4 B and C were obtained by Edelmann. To

the last detail the picture is the same as the uranium stained EM of fixed voluntary muscle shown as Figure 4 A. Thus clearly K^+ does not distribute evenly in the cell as demanded by the membrane-pump theory but is localized.

As mentioned above these findings have been completely corroborated in two other laboratories with three different additional different methods (Edelmann, 1977, 1978 b, 1980; Ling, 1977 b). Other evidence were also provided to demonstrate that K^+ does not merely exist as free-floating counterion to some fixed anionic site but is, one ion-to-one site specifically (Ling, 1977 b, c).

adsorbed

Evidence for the Existence of the Bulk of Cell Water in the State of Polarized Multilayers

The establishment of the adsorbed state of cell K^+ demands that the cell water cannot be normal for the following reason. The resting cell is in equilibrium with what is in essence a 0.1 M NaCl solution in which Na^+ and Cl^- are completely dissociated and water is in the normal liquid state. In the past, the fact that cell K^+ salt also exists at a concentration roughly 0.1 M led to the wide belief that K^+ , like Na^+ outside the cell, must also be free. Thus osmotically, the inside of the cell is a "duplicate" of what is outside the cell, both representing a simple 0.1 M salt solution. The establishment that the intracellular cation K^+ is not free but adsorbed has changed the picture completely. That is, if K^+ is adsorbed but water is free, then the osmotic activity inside and outside the cell would be quite different. Water will rapidly move from the cell to the outside and the cell would shrink drastically. Since this does not occur, something else must lower the water activity inside the cell. According to the AI Hypothesis, it is the matrix protein chains that reduce the water activity by polarizing it in multilayers. This is diagrammatically illustrated in Figure 5.

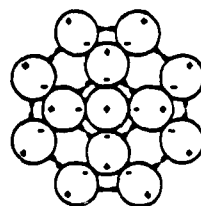
To test this theory, one must be able to prove that outside the living cells, extended protein chains can indeed polarize water in multilayers. A corollary of this theory is that when proteins exist in its native globular state in which the backbone NHCO groups are locked in α -helical or β -pleated sheet H-bonds there would be little or no multilayer polarization.

To test the multilayer polarization, we rely on the characteristic of water in polarized multilayers as suggested in the AI Hypothesis, i.e., water in this state has reduced solubility for large and complex molecules. The top section of Table I shows that when 20% aqueous solutions of 13 native proteins were introduced into 1/4 inch dialysis tubing and dialyzed against 1.5 M Na_2SO_4 solution for a time period many times longer than those needed to reach equilibrium, the Na^+ concentration inside the protein solute and in the outside medium are indeed mostly nearly equal. In other words, the apparent equilibrium distribution coefficients of Na^+ , the g -values are equal to or close to unity (Ling, Ochsenfeld et al., 1980).

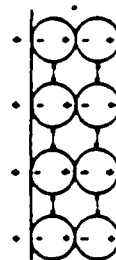
In contrast to the near unity g -value of these proteins, the g -value of Na^+ in a gelatin solution is totally different. It has a value of only 0.537. In other words, about half of the water is strongly affected by the presence of gelatin. This low g -value produced by gelatin is in full accord with the polarized multilayer theory because the presence of the triad, glycine-proline-hydroxyproline in gelatin (which is denatured collagen) prevents the formation of α -helical structure. The chain-to-chain H bonds in native collagen are largely dissolved during the denaturing process, leaving much of the backbone NHCO groups directly to bulk water, producing the expected and observed g -value lowering.

That the gelatin effect on water solvency is not due to the gel state is supported by the even more pronounced effect in

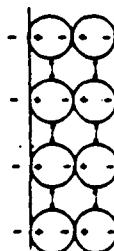
— REPULSION
— ATTRACTION



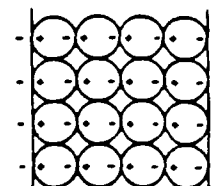
A P-TYPE



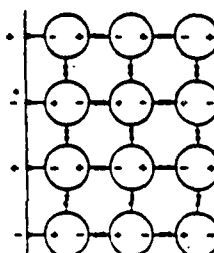
B P-TYPE



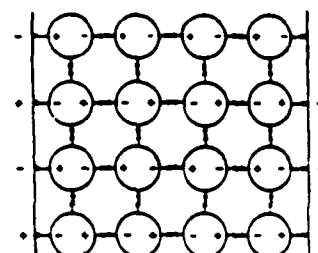
C N-TYPE



D N-P TYPE



E NP TYPE



F NP-NP TYPE

Figure 5 - Effect of charged site distribution on the stability of polarized multilayers of water molecules: — repulsion; attraction — Unstable multilayers are produced in surfaces because of lateral repulsion between molecules in the same layer: (A) P-type site, charged positive; (B) P-type site, uniformly positive; (C) N-type site, uniformly negative. Stable, deep layers of water result from lateral cohesion; (D) N-P type, P-type surface placed face to face with N-type surface; (E) NP-type surfaces with alternating positive and negative sites; (F) two NP-type surfaces placed face to face (greatest stability). (Ling, 1972) by permission of Wiley, Interscience.

lowering the g -value for Na_2SO_4 of polyvinylpyrrolidone and of poly(ethylene oxide). Neither forms gel or coarcescates under the conditions of the experiments nor can either one form intra- or intermolecular H-bond due to the lack of H-donating groups.

The pronounced effect of poly(ethylene oxide) on water solvency is most remarkable. Its extremely simple structure and the fact that its methylene and trimethylene analogues do not even dissolve in water and the copolymer of the methylene and trimethylene oxide with ethylene oxide have decreasing solubility in proportion to the percentage of these non-water soluble polymers (Stone and Stratta, 1967). All these led to the conclusion that in order to produce the effect on water solvency, the basic requirement is the presence of a matrix of chains carrying oxygen atoms at distances approximately that of two water diameters.

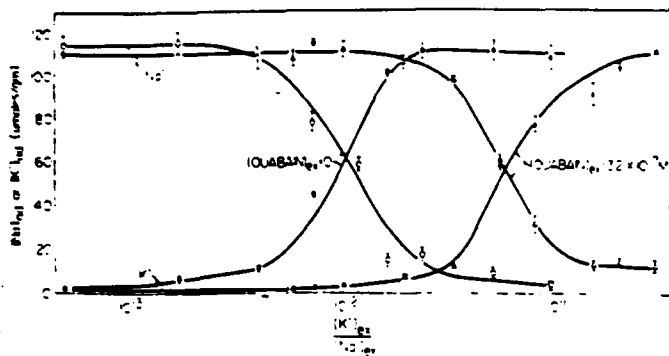


Figure 8 - Effect of Ouabain (3.2×10^{-7} M) on the Equilibrium distribution of K^+ and Na^+ ion. Curves with open (Na^+) and filled (K^+) circles were equilibrium data from muscles not treated with ouabain. The point of intersection gives a K_{Na-K} of 100. In muscles treated with ouabain (3.2×10^{-7} M), K_{Na-K} of 100. In muscles treated with ouabain (3.2×10^{-7} M), K_{Na-K} shifted to 21.7, from Ling and Bohr (1970) by permission of Physiol. Chem. Phys.

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where $-\frac{\gamma}{2} > 0$. Adsorption of this latter type is called auto-

cooperative which signifies that each time Site 2 adsorbs a K^+ , it will make the two neighboring sites prefer K^+ more than if Site 2 adsorbs a Na^+ . It is autocoooperative interaction that accounts for the kind of sigmoid shaped adsorption isotherm in oxygen uptake of red cells as well as in K^+ (and Na^+) uptake in a variety of living cells studied (Figure 8, curves labeled zero ouabain).

The sigmoid-shaped adsorption isotherm means that with the initial change of the ratio of external K^+ and Na^+ , little happens until threshold is reached at which point a small further increase of the $[K^+]_o / [Na^+]_o$ ratio tips the sites from an all Na^+ state to an all K^+ state. Similar sensitivity to small changes of oxygen tends to permit red cells effectively to serve its function of oxygen transport. The $Na^+ \rightarrow K^+$ exchange curve, has the same sigmoid shape as oxygen uptake curve of red blood cells. Only here this sigmoidity as such does not serve an obvious purpose because muscle cells are stationary and are not involved in transporting of K^+ or Na^+ between different loci of the animal body. Indeed the concentration ratio of $[K^+]_o / [Na^+]_o$ in the natural environment of most living cells including muscle are constant. Here somewhat more complicated mechanism operates involving the cardinal adsorbents.

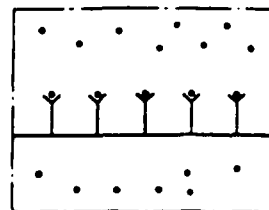
As shown in Figure 9A shifting between the two states i and j (e.g., K^+ state and Na^+ state) can be produced by varying the ratio of K^+ (i) and Na^+ (j) in the external medium. In figure 9B shifting between the two states occurs as a result of the adsorption or desorption of the cardinal adsorbent, labeled C.

From the preceding section, I have shown how in the AI Hypothesis, the healthy resting cell is poised at a metastable equilibrium state at which the cooperative assembly of protein-water and ions are such that ATP must be present at a defined level and amply replenished following its physiological degradation, and that K^+ (and not Na^+) must occupy most of the β - and γ -carboxyl groups involved and that water be maintained in the state of polarized multilayers.

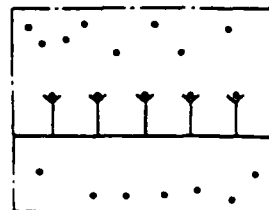
Figure 10, shows how the level of ATP adsorbed and maintained in the cell critically determines the level of K^+ in a medium containing the usual amount of K^+ and Na^+ (Ling and Ochsenfeld, 1973).

A

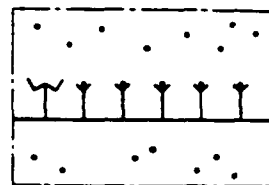
i STATE



j STATE



i STATE (CARDINAL ADSORBENT OFF)



j STATE (CARDINAL ADSORBENT ON)

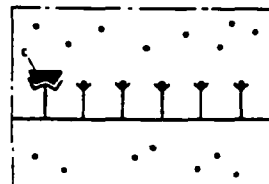


Figure 9 - A. Cooperative shifts between i and j states due to a change in the relative concentration of the i and j solutes in the environment. B. Cooperative shift between i and j state due to adsorption/desorption of cardinal adsorbent in an environment with unchanging i and j concentrations. (By permission of Annals of New York Academy of Sciences).

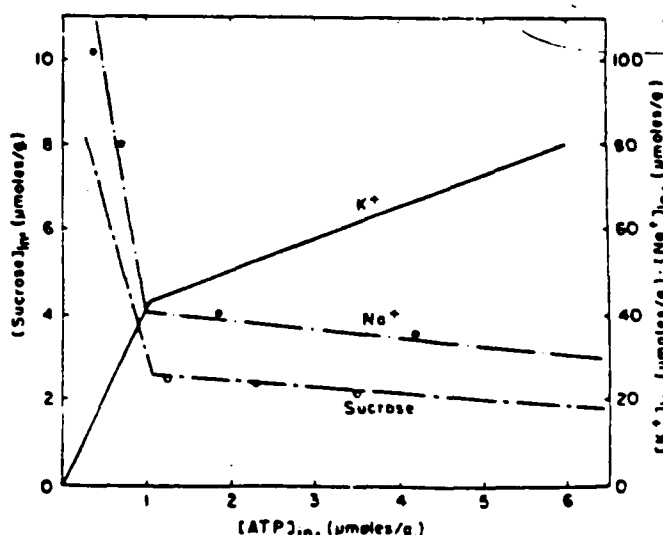


Figure 10 - Diagrammatic illustration of the variation of the K^+ , Na^+ , and sucrose concentrations in frog sartorius muscles losing ATP by the action of 0.1 mM IAA at $0^\circ C$. (Ling and Ochsenfeld, 1973) (by permission of the Ann. NY Acad. Sciences).

The pore diameters of the "active" layer of cellulose acetate membranes have been measured and shown to be 44 Å (Schultz and Asunmaa, 1969) which is many times wider than the diameter of sucrose (9.4 Å) to which it is virtually impermeable. Clearly like Traube's copper ferrocyanide gel membrane the selective permeability of cellulose acetate membrane is not due to an atomic sieve either. According to the AI Hypothesis and experimental facts, polarized water has solubility for solutes roughly in inverse proportion to molecule size and complexity. Thus a parallel reduction in diffusion coefficient in the polarized water then accounts for, in my opinion, the semipermeable selective permeability of the cellulose acetate as well as Traube's copper ferrocyanide gel membrane.

A major advantage offered by the polarized water model is that it offers a rational basis for the cell permeability to ions and nonelectrolytes as well as water to be under the control of hormones, and drugs known to affect cell permeability by interaction with cardinal sites on the water polarizing surface proteins.

Cooperativity and the Maintenance of the Normal High Energy Resting State of Living Cells

An important and distinguishing feature of the protein-ion-water system of the living cells is its internal coherence. That is to say, the system behaves as a single unit and often in all or none manner. According to the AI Hypothesis, this coherence is the result of what, in statistical mechanical terms is called cooperative phenomenon (Ling, 1962, 1969, 1980).

Cooperative phenomenon arises from the presence of interaction between neighboring elements of the assembly. Thus a piece of soft iron behaves cooperatively. The individual elements of iron atoms (domains of atoms) are each a little magnet. Under normal conditions these little magnets are randomly oriented. Thus as a whole the soft iron is not a magnet. However, if this soft iron is brought into contact with a powerful magnet, then these individual domains become oriented in one direction and the whole piece now behaves like a magnet. Here the cooperative behavior is the consequence of magnetic interaction among the individual domains.

According to the AI Hypothesis, this basic near neighbor interaction in the living cells is an electrical polarization or inductive effect. Inductive effect through the partially resonating polypeptide chains provides basis for cooperativity and the sensitivity of one segment of the protein chain to changes occurring at any region (allosteric interaction); inductive effect also underlies the multilayer polarization of layers of water molecules.

Familiar cooperative behavior in living system is the oxygenation of hemoglobin. Here the uptake of oxygen with increasing oxygen tension does not follow the usual hyperbolic pattern of an ordinary (Langmuir) type of adsorption. Rather it is sigmoid shaped and has been often described by a purely empirical equation, due to Hill (1910).

In 1964 Yang and Ling (Ling, 1964), using statistical mechanical method, or more specifically the one-dimensional Ising method, derived a cooperative adsorption isotherm based on a model of linear chains of sites each of which may have the option of two alternative adsorbents (e.g., oxygen molecules or H₂O molecules; K⁺ or Na⁺, etc.).

Since it is now firmly established that the bulk of intracellular K⁺ in muscle cells is adsorbed and it has already been established that this K⁺ can be quantitatively replaced by Na⁺ by merely lowering the K⁺ level in the incubation medium, it seems reasonable that here we are dealing with an exchange-adsorption phenomenon. As such, one can apply the Yang-Ling isotherm to describe the adsorption (Ling and Bohr, 1970).

$$[K^+]_{ad} = \frac{[f]}{2} \left(1 + \frac{\xi - 1}{\sqrt{(\xi - 1)^2 + 4\xi \exp(\gamma/RT)}} \right) \quad (1)$$

where $[K^+]_{ad}$ is the concentration of adsorbed K⁺ and is in units of moles per kilogram of fresh cells. $[f]$ is the concentration of K⁺ (and Na⁺) adsorption sites, which according to the AI Hypothesis, are the β- and γ-carboxyl groups due to aspartic and glutamic residues in the proteins. R and T are the gas constant and absolute temperature respectively.

The key variables are ξ and $-\frac{\gamma}{2}$.

ξ is defined as follows for the K⁺ - Na⁺ adsorption.

$$\xi = \frac{[K^+]_{ex}}{[Na^+]_{ex}} \quad K_{Na-K}^{oo}$$

where $[K^+]_{ex}$ and $[Na^+]_{ex}$ are the equilibrium concentration of K⁺ and Na⁺ in the external medium respectively. K_{Na-K}^{oo} is the intrinsic equilibrium constant for the $Na^+ \rightarrow K^+$ exchange and is a pure number. It is very important to note that the same change of ξ can be achieved by changing either the ratio of external K⁺ and Na⁺ concentration $[K^+]_{ex}/[Na^+]_{ex}$ or by changing

$-\frac{\gamma}{2}$, the nearest neighbor interaction energy describes the adsorption. If $-\frac{\gamma}{2}$ is negative, it means that the adsorption

of, say, a K⁺ at one site (Site 2) makes the two neighboring sites (Site 1 and Site 3) prefer Na⁺ more, than if Site 2 adsorbs a Na⁺. This type of cooperative interaction, called heterocooperative, is of lesser importance than the case

As mentioned above the critical parameters are ξ and $-\frac{\gamma}{2}$

ξ . The same effect on the adsorption can be achieved by either varying the $[K^+]_{ex}/[Na^+]_{ex}$ ratio or by changing the value of K_{Na-K}^{oo} . According to the AI Hypothesis, this change of K_{Na-K}^{oo} is induced by a propagated inductive effect (or indirect F-effect) in consequence of the desorption or adsorption of the cardinal adsorbent.

The cardinal adsorbent known to have such an effect includes Ca⁺⁺, ouabain and ATP. Figure 8 illustrates the effect of 3.26×10^{-7} M ouabain. Note that the K⁺ as well as K⁺ curves simply moved along the axis without significant distortions as a result of the ouabain action. Indeed at a fixed ratio of $[K^+]_{ex}/[Na^+]_{ex}$ corresponding to that of the plasma, the effect of ouabain was to shift the sites from all adsorbing K⁺ to all adsorbing Na⁺ a result of a decrease of K_{Na-K}^{oo} . Removal of Ca⁺⁺ and ATP have qualitatively similar effect as the addition of ouabain in lowering the value of K_{Na-K}^{oo} and hence ξ .

These findings once more emphasize that the normal high energy resting state involves (1) ATP adsorbing at its cardinal sites, (2) K⁺ adsorbing on the β- and γ-carboxyl groups primarily at the A band and Z-lines, and (3) water existing in a state of polarized multilayers with low solubility for Na⁺ etc.

A theoretical Basis for Preserving the Healthy Resting Living State of Living Cells by Increasing the K⁺ Concentration and by Decreasing Na⁺ Concentration in the Plasma or Bathing Medium When Adverse Condition Significantly Interferes with the Supply of ATP.

Figure 6 shows further that while native globular proteins do not significantly affect water solvency for Na_2SO_4 , denatured proteins whose secondary structure was disrupted (by urea, or guanidine HCl) develop similar reduced solvency for probe molecules sugar and glycine (at near neutral pH). Sodium dodecyl sulfate (SDS) and n-propanol known to disrupt only tertiary protein structure had no effect.

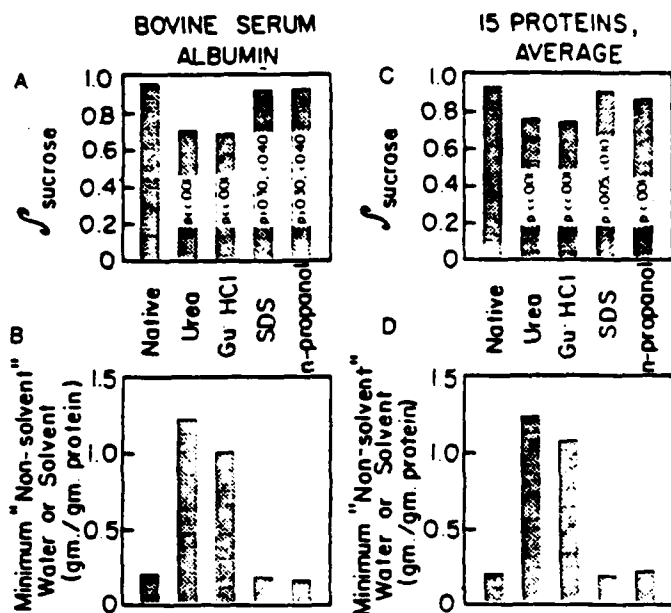


Figure 6: The q -values of sucrose (A and C) and the apparent minimum "non-solvent" water (B and D) of native and denatured proteins. C and D represent the averages of 15 proteins studied: actin, albumin (bovine), albumin (egg), chondroitin sulfate α -chymotrypsinogen, edestin, fibrinogen, γ -globulin, hemoglobin, β -lactoglobulin, lysozyme, myosin, trypsin, trypsin inhibitor, and histone. Values for the native and urea denatured states were determined from all 15 proteins, guanidine HCl values from proteins 2-11, 13, 14. SDS and n-propanol values from proteins 2-11. No significant q -value difference was observed in the native protein value whether it was determined from 15, 12, or 10 proteins. Incubating solutions contained Na_2SO_4 (100 mM), glycine (10 mM), sucrose (10 mM), and MgCl_2 (10 mM). In addition, urea (9 M) and guanidine HCl (6 M), sodium dodecyl sulfate (0.1 M), and n-propanol (2 M) were present as indicated. Incubation at $25 \pm 1^\circ\text{C}$ lasted from 28 to 96 hours, a sufficient time to establish equilibrium. The test tubes were shaken (30 excursions per min., each excursion measuring 3.4 inch). Water contents were assayed by 3 different methods (see text); sucrose was labeled with ^{14}C or ^3H ; extracts were assayed with a β -scintillation counter. (Ling et al., 1980) by permission of Physiol. Chem. Phys.)

These findings showed that extended protein chains and even the simpler oxygen-containing chains can indeed significantly reduce the solvency for Na^+ , sucrose and glycine, solute usually excluded to various extents by living cells. The data also show clearly that proteins can exist in either a water solvency affecting state and a wayrer, solvency indifferent state.

Detailed calculations of the number of water molecules involved for oxygen site shows multilayer polarization (Ling, Ochsenfeld et al., 1980). The question may be raised, "Is the number of water molecules whose solvency has been affected large enough to compare with the number required to account

for the solute exclusion in living cells"? The answer is, there is still some significant difference due to the disordered arrangement of these polymers and proteins in the solution form. In the cell environment, the matrix chain can be expected to be more orderly arranged. An orderly arrangement of the water polarizing chain, favors effective polarization. Another point worth pointing out is that the number of water molecules layers to be polarized in living cells is not nearly as extensive as some readers wrongly construed from the AI Hypothesis. In general one may estimate that the chain-to-chain distance to be no more than 10 water molecules apart. Thus counted from the basis of one chain alone, the layers polarized are only some 5 or less water molecules deep.

Going back to gelatin, one recalls how 120 years ago, Graham clearly recognized the uniqueness of gelatin and named this condition of matter as gelatin-like or colloidal. No molecular interpretation of this fundamental uniqueness of gelatin has so far been offered. The present discourse presents an interpretation of the colloidal state as that condition in which the solvent, water is polarized in multilayers.

Polarized water at the Cell Surface in lieu of Lipid Layers as the Seat of Semipermeability

I have already reviewed the recent evidence against the lipoidal membrane theory. What then comprises the continuous selective, semipermeable barrier at the cell surface?

According to the AI Hypothesis, it is water in the state of polarized multilayers. In support of this view, it was shown that a model membrane containing polymer oriented water (cellulose acetate membrane) strongly resembles living cell membrane (inverted frog skin in its selective permeability to nonelectrolysis and water). The permeability of 11 hydroxylic compounds (at three temperatures) through these membranes are not just well correlated ($r = +0.96$) but actually show near perfect correspondence (Ling, 1973) (Figure 7): the slope of the best fitting curve is 0.99.

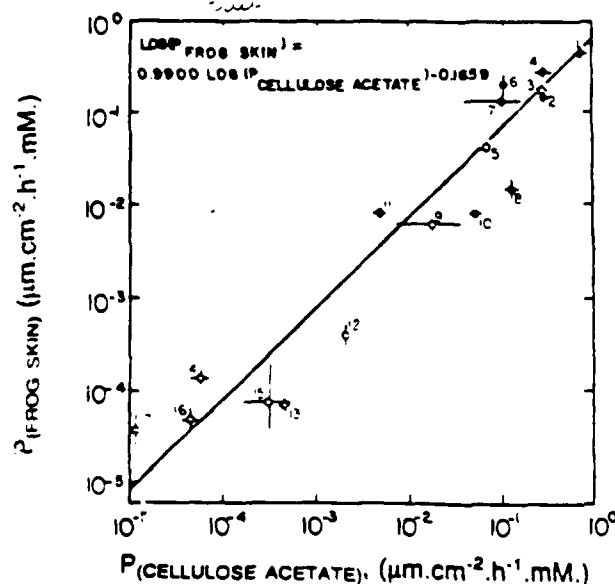


Figure 7: Plot of the permeability to 11 hydroxylic compounds ranging from water (1) to sucrose (17) at 3 different temperatures (0° , 4° , 25°C) of reversed frog skin against the permeability of heat-treated cellulose-acetate membrane. Straight line described by equation shown in graph was obtained by the method of least square (from Ling, 1973 by permission of Biophysical Journal).

Thus as ATP becomes lower the cell system will tend to be tipped over to the lower energy state where Na^+ is adsorbed and at least part of the cell water depolarized which then also takes up more Na^+ as the q -value for Na^+ rises. Along with this, the cell also undergoes swelling due to the enhanced affinity for Na^+ at the fixed anionic site under this condition and the dissociation of the restraining salt linkages essential for the maintenance of normal cell volume (Ling and Peterson, 1977).

The best way to restore the system to health would be to restore ATP to its normal value. This, however, may be difficult if impairments of the metabolic chains are not within reach of clinical management. In other words $K_{\text{Na}^+ \rightarrow \text{K}^+}$ cannot be kept at its normal high value.

Fortunately the critical determining factor between health or debilitation is not $K_{\text{Na}^+ \rightarrow \text{K}^+}$ but ξ . ξ can be kept at normal level even if $K_{\text{Na}^+ \rightarrow \text{K}^+}$ has been seriously diminished if one increases the ratio of $[\text{K}^+]_{\text{ex}}/[\text{Na}^+]_{\text{ex}}$ in the blood plasma. Such a change

of plasma level of K^+ and Na^+ may be achieved for example, by lowering dietary Na^+ uptake and by enhancing K^+ uptake or by infusion with the solutions of proper K^+ and Na^+ concentrations.

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* That the Association-Induction Hypothesis may offer an interpretation for beneficial effect of low Na^+ and high K^+ diet was pointed out by Cope in 1977.

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SUMMARY

*The Association-Induction hypothesis.
A theoretical Foundation Provided by possible beneficial Effects of a low Na, high K diet and other similar Regimens
in the Treatment of Patients suffering from debilitating illnesses*

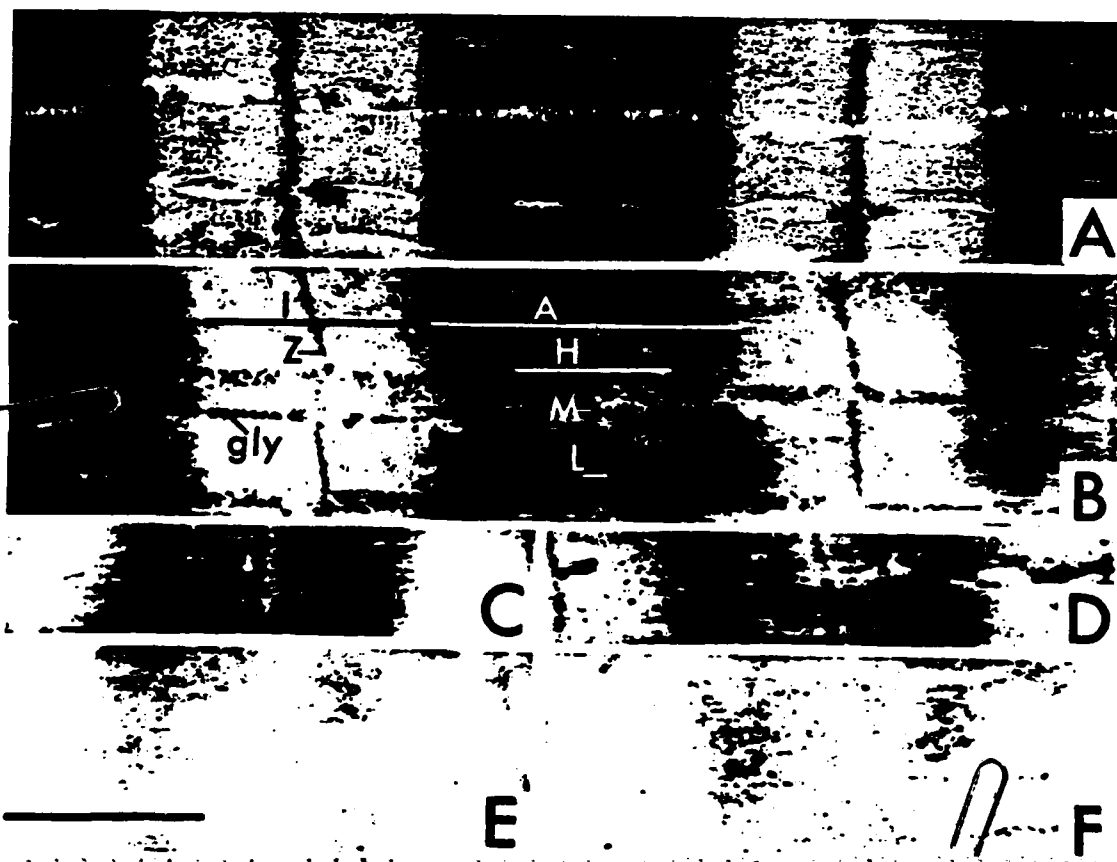
G.N. LING

Agressologie, 1983, 24, 7 :

The article began with a brief review of the history of cell physiology, followed by a summary of some compelling reasons why the conventional widely taught membrane-pump hypothesis is no longer tenable. An alternative theory, the Association-Induction Hypothesis was presented as well as the theoretical basis this hypothesis offers for possible beneficial effects of a low Na⁺ high K⁺ diet or other similar regimens for the treatment of patients suffering from debilitating illnesses.

Figure 4.

Electron micrographs of frog sartorius muscle. (A) Muscle fixed in glutaraldehyde only and stained with uranium by conventional procedure. (B) EM of section of freeze-dried Cs^+ -loaded muscle, without chemical fixation or staining. (C) Tl^+ -loaded muscle without chemical fixation or staining. (D) Same as C after exposure of section to moist air, which causes the hitherto even distribution of thallium to form granular deposits in the A band. (E) Section of central portion of B after leaching in distilled water. (F) Normal "K-loaded" muscle. A: from Edelmann, unpublished. B to F from Edelmann (1977) (By permission of Physiol. Chem. Phys.)



STUDIES ON THE PHYSICAL STATE OF WATER IN LIVING CELLS AND
 MODEL SYSTEMS. I. THE QUANTITATIVE RELATIONSHIP BETWEEN
 THE CONCENTRATION OF GELATIN AND CERTAIN OXYGEN-
 CONTAINING POLYMERS AND THEIR INFLUENCE UPON THE
 SOLUBILITY OF WATER FOR Na^+ SALTS

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• *The quantitative relationships between the concentrations of solutions of gelatin, polyvinylpyrrolidone, poly(ethylene oxide), polyvinylmethylether, and poly(ethylene glycol), and their ability to reduce the solubility of water for Na citrate are presented. The data in general are in harmony with the polarized multilayer theory of protein (and polymer) dominated water in vitro and in living cells.*

INTRODUCTION

According to the membrane-pump theory, cell water and ions are largely free as in a dilute aqueous salt solution. To explain the asymmetrical distribution of Na^+ and other permeant solutes, membrane pumps were postulated. Extensive evidence now exists against the membrane-pump theory. For example, (i) pumps require an amount of energy greater than that available;^{5,14} (ii) closed membrane sacs without cytoplasm do not generate and maintain asymmetrical Na^+ or K^+ gradients,^{9,15} while cells without functional cell membranes do;¹¹ (iii) in the membrane-pump theory, the major intracellular cation, K^+ , must exist in a free state in order to explain the maintenance of osmotic balance and the generation of a "membrane potential"; recent evidence shows that cell K^+ does not exist in a free state but is adsorbed on specific sites on intracellular proteins.^{1,2,3,9,19} While contradicting the membrane-pump theory, these findings are either in harmony with or directly support an alternative theory of the living cell, the association-induction hypothesis.^{4,5,6,7,10,12} According to this hypothesis, the high level of cell K^+ is due to specific adsorption of this cation on proteinaceous anionic sites (e.g., β - and γ -

carboxyl groups) and the low levels of Na^+ , sugars, and free amino acids reflect a unitary cause: reduced solubility in the cell water of these and other "large" and complex molecules and hydrated ions. Due to interaction with a matrix of protein chains, called the matrix proteins — which are postulated to exist throughout the cell interior of all cells — the bulk of cell water exists in the state of polarized multilayers. The postulated matrix proteins in this theory must exist in an extended conformation with their polypeptide chain NH and CO groups directly exposed to the bulk phase water, providing anchoring and polarizing sites for multiple layers of water molecules.

Recently reported studies of several model systems lend support to this view. Proteins which, for structural reasons (e.g., gelatin) or in response to secondary-structure breakers (e.g., urea, guanidine HCl), exist in an extended conformation, reduce the solvency of the bulk phase water for Na^+ salts, sucrose, and glycine. In contrast, many native globular proteins with their NH and CO groups locked in α -helical or other intramolecular H-bonds are ineffective. Several synthetic polymers resemble the postulated

matrix proteins and also carry oxygen atoms at a distance roughly equal to two water diameters from the nearest neighboring oxygen atoms and are unable to form intramolecular or intermacromolecular H-bonds. These polymers, including polyvinylpyrrolidone (PVP), poly(ethylene oxide) (PEO), polyvinylmethylether (PVME), and a number of native gums, polysaccharides, etc., also reduce water solvency for Na⁺ salts, sucrose and glycine.^{16,17} In the terminology of the association-induction hypothesis, a matrix of extended protein chains with alternately negative (N) CO and positive (P) NH sites is called an NP-NP-NP system, while a matrix of polymer chains like PVP, and PEO with only negative (N) oxygen separated from each other by vacant (O) sites is called an NO-NO-NO system.

These water-polarizing polymers provide a way to produce experimental models of water in a physical state (the state of polarized multilayers) hitherto known only in theory. The reduced solvency of water in this state is highly relevant to the physiological role of cell water in the maintenance of cell solutes at physiological levels; it also makes it possible to measure quantitatively the minimal amount of water affected by the polymer present. To date, our published data on this subject have been presented only in a piecemeal manner. The present communication presents in a systematic way the quantitative relationship between the degree of solvency change and the concentration of the water-polarizing polymer present. Results of studies of a new polymer, poly(ethylene glycol) are also presented.

MATERIALS AND METHODS

The basic method used was equilibrium dialysis by procedures described in earlier work.^{16,17} Polymer solutions (20 to 40%) were projected into 1/4 inch dialysis tubing and incubated with gentle shaking in solutions of

various concentration of Na citrate labelled with Na²². Incubation was, in most cases, at 25° ± 1°C in a constant temperature room for a length of time long enough to insure equilibrium (2 to 3 days). At the conclusion of the experiment, the labelled Na⁺ content was assayed with the aid of a γ -scintillation counter and expressed as a ratio to the labelled Na⁺ concentration in the external bathing solution, called the apparent equilibrium distribution coefficient or ρ -value.

$$\rho = \frac{\text{Na}^+ \text{ concentration in the sac}}{\text{Na}^+ \text{ concentration in the bathing solution}}$$

The ρ -value of Na⁺ (as citrate) equals the true equilibrium distribution coefficient (or q -value) of Na⁺ when there is no adsorption on or complexing of this ion with macromolecules and when the Na⁺ is entirely dissolved in the water in the sac. The water content within the sac and in the external bathing solution were determined by weighing before and after drying under vacuum in a 100°C oven for PVME, PVP, and gelatin and in a 60°C oven for PEO.

By definition, both the q -value and the ρ -value are ratios of *molar* concentrations. The molar concentration of the probe molecules or ions in the bathing solution is easily determined by dividing the measured probe content in moles by the volume of the solution. The molar concentration of the probe molecule inside the sac is less simple since the sac contains a high content of the polymer under study. The method chosen to obtain the molar concentration is to determine first the *molal* concentration of the probe by dividing the quantity of probe in moles in a sample by its water content (in liters). The molal concentration of the probe in the sac thus obtained is then converted to the molar concentration with the aid of the data given in Figure 1 which presents the molal to molar conversion ratios for the Na citrate and Na sulfate at 25°C.

The p -value determined was used to calculate the amount of Na citrate in the sac, which was subtracted from the total dry weight of the sac content to yield by difference the final polymer concentration in percentage (w/w) in the sac.

To determine the molar concentrations of Na citrate and Na sulfate prepared in molal concentrations, calibrated Babcock "milk bottles" with thin and long graduated necks were used (A. H. Thomas, Phila., PA).

Gelatin manufactured from pig and calf skin was obtained from Eastman and from Fischer Scientific Co., Phila., PA. PVP was from Sigma Chemical Co., St. Louis, MO. (Mol. wt. 360,000). PVME (Gantrez M-154) was in part a gift and purchased from GAF Corp., New York, NY. PEO (Polyox 205®) was a gift from Union Carbide, New York, NY. Na^{22} was obtained from ICN, Irvine, CA (Lots 39 and 40) and from New England Nuclear, Boston, MA (Lots 4771CG10 and 5812RG6). Poly(ethylene glycol) (Carbowax, PEG 20,000) was from Fischer Scientific Co., Phila., PA (Lot 714-714).

RESULTS AND DISCUSSION

Figures 2, 3, 4, 5, and 6 present the p -values for Na citrate in the presence of different concentrations of gelatin, PVP, PEO, PVME, and PEG respectively. The data on gelatin, PVP, PEO, and PVME are those accumulated between 1976 and 1982 and include previously published data.^{16,17} The data on PEG are new.

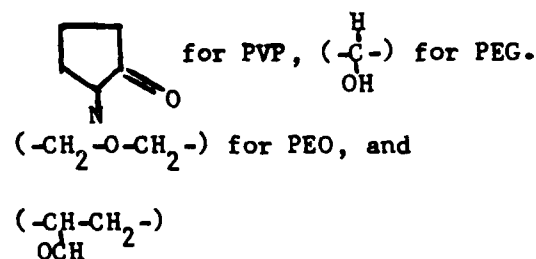
When compared on equal weight percentage basis, gelatin was the least effective in reducing the water solvency for Na citrate. The most effective are PEG and PVME with PEO and PVP falling in between PVME and gelatin.

Figures 2 to 6 provide the data to calculate the minimum number of water molecules that have been affected by interaction with the polymer at a specific concentration.

Thus, if at a polymer concentration of, say, 20% the p -value for Na citrate is 0.5, then $\frac{(1-0.2) \times 0.5}{0.2} = 2$ grams of water per gram

of dry polymer has appeared to have completely lost its solvency for Na citrate. This water has been referred to as the apparent minima "non-solvent" water (AMINOW).¹⁶

Since each of the polymers studied contain only one kind of monomeric unit and since there are good reasons to believe that the sites of polymer-water interaction are the oxygen atoms present in each monomeric unit, the minimal amount of affected water can be more meaningfully expressed in terms of the number of water molecules per oxygen atom or per monomer unit of the polymer. These monomers are



for PVME, with monomer weights of 112.15, 32.04, 44.05, and 58.08 respectively. As an example, for the calculation of the molar AMINOW, we chose a 20% PEO solution with a p -value equal to 0.5. In this solution, there are $200/44.05 = 4.54$ moles of monomers each carrying one oxygen site. The minimum number of water molecules affected by each oxygen site is then

$$\frac{55.5 \times (1-0.2) \times 0.5}{4.54} = 4.89. \text{ From the}$$

smoothed curves of Figures 2 to 6 the p -values and AMINOWS for all concentrations of PVP, PEO, PVME, and PEG are tabulated in Table I. We have no idea how many of the NHCO groups in gelatin are free

and not locked in "collagen folds" and thus made unavailable in reacting with bulk phase water. Therefore we could not make a similar estimate of AMINOW for gelatin.

Figure 7 plots the molar AMINOW at different concentrations of the polymer. Note that as the concentrations of PEO and PVME increase there is first a rapid rise of AMINOW, followed by a decline. The rise and decline are absent or less conspicuous for PEG and PVP in the concentration ranges studied.

The low AMINOW at very low PEO and PVME content suggests that the existence of oxygen atoms on the polymer chain at right distances apart is, by itself, an insufficient condition for the maximum reduction of water solvency toward Na citrate. It seems that the polymer to polymer relation may play a role in the enhancement of the water-solvency reducing effect. In the polarized multilayer theory, the role of the chain to chain interaction is to reinforce water polarization as the chain-to-chain distance decreases, diagrammatically illustrated in Figure 8. However, when the chain-to-chain distance becomes too close (i.e., polymer concentration too high), the number of water molecules with "non-solvent" properties will decrease as the water molecules polarized by one chain overlap those polarized by neighboring chains. This decreased efficiency may not only reflect duplication of the polarizing effects but may also involve mutual cancellation since in these cases all polarizing sites carry electrical charges of the same sign.

At a 7.5% concentration, the molar AMINOW of PVME reaches a figure of about 20. There are reasons to believe that under favorable conditions the actual number of water molecules that an oxygen atom of PVME can polarize may exceed 20 considerably: (1) the polymer chains in our samples must be highly random in distribution. Many oxygen sites are therefore too close to other oxygen sites, and overlap of

their polarization realms reduces the average number of water polarized by these sites. In support of this contention, Ling *et al.*^{16,17} already showed that stirring, which tends to line up linear polymer chains, decreases ρ -values. (2) The AMINOW is an estimate of the minimal number of water molecules polarized. Since it is highly unlikely that there is absolute exclusion of Na^+ from this water, the actual number of water molecules under the influence of each polymer oxygen must also, for this reason, be higher. With these considerations in mind, we are inclined to believe that under optimal conditions a PVME oxygen atom polarizes considerably more than 20 water molecules. Regardless of exactly how many more than 20 are adsorbed, there seems little question that multilayers of water are involved.

Let us next raise the question: If the optimal spatial configuration of the PVME molecules as seen in a 7.5% solution can be uniformly maintained at a higher concentration, what PVME concentration would be required to yield a q -value of 0 for Na citrate? Let us represent this concentration as $n\%$. The total number of non-solvent water would be $30 \times \frac{10n}{58.08}$ while the total molar concen-

tration of water is $(1 - \frac{n}{100}) 55.5$. All this water is nonsolvent when $(1 - \frac{n}{100}) 55.5 =$

$30 \times \frac{10n}{58.08}$. One calculates that n is equal to 9.7. Thus, ideally, a 9.7% PVME should be able to have a q -value of 0 for Na citrate. In reality, the q -value of a 9.7% PVME is not zero but about 0.45. There are no doubts that the random distribution of the chains play a role in this loss of efficiency.

As mentioned earlier, PVP, PEO, and PVME are all what are called NO-NO-NO systems. Theoretically NO-NO-NO systems are less effective than one like the NP-NP-NP system of the extended polypeptide chains. Here, the alternately positive and negative sites of CO and NH group further stabilize

the multilayer water structures.^{8,12} It may well be expected that the postulated matrix proteins in living cells may be so ordered as to avoid any wasteful overlap of water polarization as has been shown for the solutions of synthetic polymers. In this case, perhaps less than 9.7% of the matrix protein would be needed to polarize all the cell water, even if one completely ignores (i) the space-filling and water-content reducing effect of other non-matrix proteins and macromolecules in the cell; and (ii) the weak but significant water polarizing effects of some globular proteins.¹³ That only a small concentration of matrix proteins can polarize all the cell water is necessary in the polarized multilayer theory of cell water.

Finally one would like to comment on the ability of PEG to reduce water solvency. Note that in contrast to PVP, PEO, and PVME, each of the oxygen atoms of the alcoholic group of PEG is placed between two other similar oxygen atoms on adjacent carbon atoms in the $(-\text{CH}-\text{CH}-)_n$ backbone. This is in apparent contradiction to the conclusion that to achieve a solubility reducing effect, the oxygen atoms must be separated by distances roughly equal to twice the water diameters (as in the case of PVME where the oxygen atoms are attached to every other carbon atom on the hydrocarbon backbone). A possible solution to this apparent contradiction may lie in the unusual nature of the oxygen carrying groups, the alcoholic $-\text{OH}$. Thus, we suggest that PEG, instead of representing an NO-NO-NO system, actually represents an NP-NP-NP system. Here the lone pair of electrons on the oxygen atom acts as an N site very much in the same way that the oxygen atoms in the $-\text{OCH}_3$ groups in PVME. The immediately neighboring OH groups may then act as P sites analogous to the peptide NH group. It is also well known that hydroxyl groups behave as strong proton donors as well as strong proton acceptors.¹⁸ Other evidence supports the idea that PEG represents an NP-NP-NP system:

(i) the high effectiveness of PEG in reducing the solvency for Na citrate; and (ii) the failure of the AMINOW of PEG to undergo a sharp drop even after PEG concentration has reached as low as 2.5%.

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FIGURE 1. The molal-molar conversion factor for solutions of Na citrate and Na sulfate.

FIGURE 2. The apparent equilibrium distribution coefficient (ρ -value) of Na citrate between various concentrations of gelatin solutions in the bags and in the external solution (37°C). The higher temperature was to keep the gelatin in the same fluid state as in other polymers. For data at 25°C and 0°C see Ling *et al.*, 1980b.

FIGURE 3. The apparent equilibrium distribution coefficient (ρ -value) of Na citrate between various concentration of PVP solutes in the bag and in the external solution at various concentrations of PVP (25°C).

FIGURE 4. The apparent equilibrium distribution coefficient (ρ -value) of Na citrate between water in the presence of varying concentrations of polyethylene oxide in the dialysis sacs and the water in the external solutions containing no polymers (25°C). Combined results of 4 sets of experiments. Distances between bars represent twice the standard error.

FIGURE 5. The apparent equilibrium distribution coefficient (ρ -value) of Na citrate between varying concentration of polyvinylmethylether solutions in the dialysis sacs and the external solutions (25°C). The initial Na citrate (or sulfate) concentrations in the bathing solutions were from 5 mM to 1.5 M and are indicated in the figure. Combined data from 7 sets of experiments. Distances between bars represents twice the standard error. Points at low PVME concentration were given as single determinations; the great variability of the water and polymer contents made averaging of these data undesirable.

FIGURE 6. The apparent equilibrium distribution coefficient (ρ -value) of Na citrate between various concentrations of PEG solution and the external solutions (25°C).

FIGURE 7. The apparent minimal "non-solvent" water (AMINOW) to Na citrate in solutions of PVP, PEO, PVME, and PEG solutions of different concentrations. The molar AMINOW is given in average number of H₂O molecules per oxygen atom in the polymer.

FIGURE 8. Diagrammatic illustration of the effect of decreasing distance between extended protein chains or model polished glass surfaces. N represents negatively charged sites and P positively charged sites. NP-NP system represents two juxtaposed surfaces (e.g., polished

glass) containing N and P sites with regular spacing like a checkerboard. NP-NP-NP system represents an equivalent matrix of linear chains carrying N and P sites at regular intervals separated from each other by distances roughly that of one water diameter. N and P may represent the CO and NH groups of an extended protein chain.

TABLE I. The p -value and the molar apparent minimal "non-solvent" water for Na citrate in solutions of PVP, PEO, PVME, and PEG of different concentrations.

Polymer Content (% w/w)		2.5	5	7.5	10	15	20	30	40	50	60
Water Content (% w/w)		97.5	95	92.5	90	85	80	70	60	50	40
PVP	$p\text{Na-Cit}$	—	—	—	0.80	0.72	0.63	0.42	0.30	0.13	0
	AMINOW (average number of H ₂ O molecules per oxygen atom)	—	—	—	11.2	9.77	9.21	8.43	6.52	5.40	4.15
PEO	$p\text{Na-Cit}$	—	—	0.87	0.73	0.51	0.39	0.22	0.13	0.08	—
	AMINOW (average number of H ₂ O molecules per oxygen atom)	—	—	3.92	5.90	6.80	5.96	4.45	3.19	2.25	
PVME	$p\text{Na-Cit}$	—	0.89	0.50	0.45	0.39	0.34	0.22	0.11	0.05	—
	AMINOW (average number of H ₂ O molecules per oxygen atom)	—	6.70	19.9	16.0	11.2	8.52	5.87	4.30	2.96	
PEG	$p\text{Na-Cit}$	0.75	0.65	0.56	0.49	0.38	0.29	0.16			
	AMINOW (average number of H ₂ O molecules per oxygen atom)	13.5	11.8	9.66	8.17	6.26	5.05	3.49			

STUDIES ON THE PHYSICAL STATE OF WATER IN LIVING CELLS AND
MODEL SYSTEMS. II. NMR RELAXATION TIMES OF WATER PROTONS
IN AQUEOUS SOLUTIONS OF GELATIN AND OXYGEN-CONTAINING
POLYMERS WHICH REDUCE THE SOLVENCY OF WATER FOR Na^+ ,
SUGARS, AND FREE AMINO ACIDS

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• This communication reports our study of the NMR relaxation times, T_1 and T_2 of water protons in aqueous solutions of bovine serum albumin, gelatin, polyvinylpyrrolidone, poly(ethylene oxide), and polyvinylmethylether over a wide concentration range. In contrast to solutions of gelatin and bovine serum albumin, the T_1/T_2 ratio of the three synthetic polymers are close to unity over the entire range studied. When combined with earlier-reported data of water made "non-solvent" to Na salts, the present data provided the basis for calculating the T_1 and T_2 as well as the rotational correlation time τ_c of the "non-solvent" water. It was shown that only a modest reduction by a factor of about 3 of τ_c is enough to produce water that is "non-solvent" for Na citrate and sulfate. The new data reconciles NMR data of living cells with the theory of the cell water given in the association-induction hypothesis. The variability of τ_c of "non-solvent" water also offers explanations of apparently conflicting conclusions on the physical state of cell water from dielectric measurements.

INTRODUCTION

The development of the theories and techniques of NMR spectroscopy opened ways of studying living cells never known before. One subject of interest concerns the most abundant and ubiquitous component of all living cells: water. The central question is, "Does the bulk of water in living cells exist as free water as postulated in the membrane (pump) theory?" or "Does the bulk of cell water exist in the state of polarized multilayers and suffer rotational as well as diffusional motional restriction as suggested in a more recent theory of the living cell, the association-induction hypothesis (Ling, 1965a, 1965b, 1967, 1972, 1977, 1979a, 1979b, 1981; Ling and Sobel, 1975). In testing these alternative theories, a potentially useful approach involves the measurement of the spin-lattice relaxation time of the water protons, also called T_1 , and

the spin-spin relaxation time, also called T_2 . The theory of NMR describes the magnitude of T_1 and T_2 as functions of the correlation time (τ_c) — which is the characteristic time of the rotation of a molecule or the time of its diffusion into its next position (Bloembergen et al., 1948; Kubo and Tomita, 1954; Solomon, 1952). When water molecules turn freely as in liquid water, τ_c of the water protons is short (2.6×10^{-12} sec.) (25°C) (Smith and Powles, 1966); in this case, T_1 and T_2 of the water protons are nearly equal (see below). When water molecules freeze into ice, the τ_c becomes much longer. T_2 then becomes much shorter than T_1 .

Since (Odeblad et al., 1956) made NMR measurements of water protons in living cells, much information on this subject has accumulated (for review, see Hazlewood, 1979). In general, it has been found that T_1

of water protons in living cells is considerably longer than T_2 , often by a factor of about 10. This difference between T_1 and T_2 by itself may be taken to indicate an "ice-like" or quasi-crystalline structure of cell water. However an examination of other characteristics of cell water tells a different story. Thus, the self-diffusion coefficient of water in living cells differs as a rule from that of normal liquid water by a factor not more than 2 (Ling et al. 1967, Abetsedarskaya et al. 1967, Mild et al. 1972, Finch et al. 1971).¹³ For review see Hazlewood, 1979¹⁶; while in ice, the self-diffusion coefficient of water is one million times slower than that of normal liquid water (Eisenberg and Kauzmann, 1969¹⁰). Therefore little doubt exists that cell water cannot be literally ice-like.

Taking into consideration these and other findings, some workers in this field have adopted the view that the bulk of cell water is simply normal liquid water. The reduced T_1 and T_2 observed are seen as the consequence of rapid exchange of this normal liquid water with one or more small fractions of rotationally bound or otherwise effectively relaxing water molecules with much shorter T_1 and T_2 (Zimmerman and Brittin, 1957, Cooke and Kuntz, 1974¹⁷).

Now if the bulk of cell water is truly normal liquid water, many "pumps" must be postulated for living cells (see Ling, Miller and Ochsenfeld, 1973³²) in order to account for the asymmetrical distribution of sugars, Na^+ (vs. K^+), etc., between living cells and their environments. Yet it has been shown that the cell does not have enough energy to cope with even one of the postulated pumps (Ling, 1962, Chapt. 8, 1965a, 1980a²⁵). Serious and probably insurmountable as this objection is, it is but one of a lengthening list of evidence against the membrane-pump theory (Ling, 1977, 1983a, 1983b³³). Clearly some other explanations must be found in order to explain all the relevant data.

In the early 70's when the NMR investigation of cell water was most vigorously pursued, the polarized multilayer theory was not well known, nor was there a verifiable "test tube" model for this theoretically postulated state of water. Fortunately the condition has improved. First it was shown on theoretical grounds that the polarized multilayer theory does not imply a high degree of motional restriction of the water involved. Indeed, even large and complexed hydrated molecules and ions like Na^+ only need to be reduced in their motional freedom by a factor of 10 to account for the observed degree of Na^+ exclusion (Ling, 1979a²⁶). Simple model systems have also been found in which the bulk of the water does indeed exhibit properties predicted by the polarized multilayer theory, including the ability to exclude Na^+ , sucrose, and free amino acids (Ling et al. 1980a, b²⁷). One recalls that it is the maintenance of low levels of these solutes in cell water that led first to the introduction of the membrane-pump theory and then the polarized multilayer theory of cell water (Ling, 1965b²⁸).

The choice of solvency reduction as the criterion for the recognition and detection of water in the state of polarized multilayers offers various advantages. Direct relevance to cell physiology is one. Another advantage lies in the quantitative information about the affected water that the solute distribution study provides. That is, the true equilibrium distribution coefficient of a probe molecule (or q -value) or the apparent equilibrium distribution coefficient (or p -value) (Ling and Sobel, 1975³⁴), can tell us quite unambiguously the minimal amount of the water that has been altered by the condition or substance that brings about the multilayer polarization. Studies of a family of polymers and proteins has shed light on the mechanism whereby these polymers and proteins change the properties of water (Ling et al. 1980a, b²⁷). Thus all the effective ones contain oxygen atoms at distances roughly equal to

that of two water diameters apart. They are all "extended": the oxygen atoms are not tied up in one or another form of macromolecular H-bonds and thus are available to interact with the bulk phase water.

Among the polymers that affect water properties, gelatin has a long history, dating back to before Thomas Graham (1861) who used it to represent colloids. This unusual protein falls into the category of an NP-NP-NP system (Ling, 1972): its positively charged polypeptide NH (P) and negatively charged CO (N) groups are held in an extended and exposed condition due to the possession of many helix-breaking amino acid residues: glycine, proline, and hydroxyproline (Veis, 1964; Chou and Fasman, 1974). Other simpler water-polarizing polymers include polyvinylmethylether (PVME), polyvinylpyrrolidone (PVP), and poly(ethylene oxide) (PEO); all belong to what is called an NO-NO-NO system as these polymers possess no P sites but only N sites in the form of oxygen atoms with its negatively charged two lone pairs of electrons. The sites between each pair of N sites are vacant and are referred to as O sites.

Following the recognition of the water-property-altering power of these polymers, we (and others) conducted investigations of several other physico-chemical attributes of water in this unusual physical state. Besides its solvency-reducing effects already mentioned (Ling et al., 1980a, b; Ling and Ochsenfeld, 1983), other studies include the osmotic activity (Ling, 1983a); swelling and shrinkage (Ling 1980b, 1983a); freezing and thawing (Zhang and Ling, 1983); and quasi-elastic neutron scattering (Rorschach, 1983). This communication adds the results of our measurements of the NMR relaxation times T_1 and T_2 of similar polymer- and gelatin-dominated water and of water in solutions of a protein that does not have the solvency-reducing power, native bovine serum albumin (BSA). A brief note was published earlier (Ling and Murphy,

1982).

MATERIALS AND METHODS

Materials. Polyvinylmethylether (PVME) (Gantrez M-154[®]), Batch 185, was from GAF Corp., NY; poly(ethylene oxide) (PEO), (Polyox-205), from Union Carbide, NY; polyvinylpyrrolidone, PVP-360, from Sigma Chemical Co., St. Louis, Lot 57C-0071; and gelatin (Type I, approx. 300 Bloom), (swine skin) also from Sigma.

NMR relaxation time measurements. Water proton NMR relaxation times T_1 and T_2 were measured with a coherent CPS-2 NMR pulse spectrometer (Spin Lock, Ltd., Port Credit, Canada) operating at a resonance frequency of 17.1 MHz. T_1 was determined with a 180° - τ - 90° pulse sequence; care was taken to insure reestablishment of equilibrium between readings by allowing at least five times T_1 in between readings (see Farrar and Becker, 1971). T_2 was determined with Carr-Purcell spin echo methods. The sample temperature was $25^\circ \pm 1.0^\circ\text{C}$.

Preparation of paramagnetic ion-free polymers. All glassware -- including drying vessels and transfer pipets -- which came into contact with polymers, were acid washed (concentrated HCl or *aqua regia*) and triple rinsed in distilled water. A 5% polymer solution was prepared by floating dry polymer over water in a wide vessel and allowing it to shake gently until dissolved. Washed Chelex 100 resin (Bio-Rad, Richmond, Calif., 100-200 mesh, sodium form) was added to the solution and allowed to shake with the polymer solution until the solutions were free of paramagnetic ions (2-8 days depending on polymer). To assay for paramagnetic impurities the solutions were dried and the dry residues ashed at 600°C in a muffle furnace. The T_1 of a dilute acid extract of the ashes when compared with that of the acid itself determines the presence or

absence of the paramagnetic impurities. No attempt was made to remove oxygen in the samples.

RESULTS

Bovine serum albumin and gelatin. Figure 1 gives illustrative examples of plots of $\ln(A_\infty - A_\tau)$ against varying values of τ for solutions of purified bovine serum albumin (BSA), gelatin as well as the polymers PEO, PVP, and PVME. A_∞ is the initial amplitude of the free induction decay signal following the 90° pulse at time τ . A_∞ equals A_τ when $\tau \rightarrow \infty$. As a rule, the data can be adequately described by a single slope, equal to $-(T_1)^{-1}$. Figure 2 illustrates the Carr-Purcell plots for the determination of T_2 . The logarithm of the echo height at time t , $\ln A_t$, is linearly related to t also with a single slope, equal to $-(T_2)^{-1}$. With these methods the data on T_1 and T_2 were obtained for solutions of BSA and gelatin at different concentrations and shown in Figures 3 and 4 respectively. The T_1 's are considerably longer than T_2 throughout the entire range of concentrations studied. The differences between T_1 and T_2 are most pronounced at the lower H₂O contents where both the T_1 and T_2 curves bend sharply. In Figures 5 and 6, the same data were plotted as $1/T_1$ and $1/T_2$ against the H₂O contents. Note that in each case the $1/T_1$ and $1/T_2$ abruptly diverge after the water content falls below a certain value: 72% for BSA and 51% for gelatin. In general these data are close to those reported earlier for isolated proteins (Cooke and Wien, 1971⁵⁴). However, quantitatively the curves for gelatin and BSA differ considerably. At the same concentrations, T_1 and T_2 of gelatin are much longer than those of BSA.

PVP, PEO and PVME. Figures 7, 8, and 9 show the T_1 and T_2 values from solutions of PVP, PEO, and PVME, respectively. The results differ markedly from those of protein solutions. First, the T_1 and T_2 curves are

much closer together. The T_1/T_2 ratios of solutions of these polymers as given in Table 2 are not that far different from pure water at several pH's in air and in nitrogen as given in Table 1 (see also Meiboon et al, 1957⁵⁵; Meiboon, 1961⁵⁶). Figures 10, 11, and 12 show nearly equal rates of spin lattice relaxation and spin-spin relaxation represented by $1/T_1$ and $1/T_2$ respectively, in marked contrast to the widely divergent $1/T_1$ and $1/T_2$ of the two proteins shown in Figures 3 and 4, Table 2.

DISCUSSION

Earlier Interpretations of NMR Data of Protein Solutions and of Living Cells. The theory of Bloembergen, Purcell and Pound (1948)⁵⁷, Kubo and Tomita (1954)⁵⁸ and Solomon (1952)⁵⁹ permitted the calculation of the relaxation rates ($1/T_1$ and $1/T_2$) as a function of τ_c at a specific field frequency. For water protons in a magnetic field of 17.1 MHz (the frequency of our instrument), the relation between τ_c , T_1 and T_2 is illustrated in Figure 13. To be noted is that T_1 goes through a minimum, the value of which varies with the field frequency. At τ_c lower than that corresponding to the T_1 minimum, T_1 and T_2 are essentially equal; above it, they diverge. As mentioned earlier, the longer T_1 than the T_2 often observed for water protons in living cells and in solutions of a variety of isolated proteins have been interpreted as due to rapid exchange between a minor fraction of water protons with a very high value of τ_c and a much larger fraction with a much lower τ_c . Thus if the rate of this exchange is much faster than the NMR relaxation rates, then the observed NMR relaxation time T_{obs} would be a single value equal to the weighted average of relaxation times in the n different populations of water protons (Zimmerman and Britten, 1957⁵⁶):

$$T_{obs}^{-1} = \sum_{i=1}^n P_i T_i^{-1} \quad (1)$$

where P_i is the probability of the water proton being found in that specific i th population with T equal to T_i . P_i is usually taken to be equal to the size of the proportion of water belonging to that population.

Cooke and Kuntz (1974) explained the observed T_1 and T_2 of water protons in isolated protein solutions at 1 to 100 MHz on the assumption that the bulk of water is normal liquid water (Type I) with a τ_c equal to 3×10^{-12} sec. in rapid exchange with one fraction of hydrated water (Type II) of an amount equal to 0.3 to 0.6 g. H_2O g. protein with a τ_c of 10^{-9} sec., and with another minute fraction (Type III) as little as 0.003 g. H_2O g. protein with a τ_c of 10^{-6} sec. They then extended this conclusion to living cells.

Cooke and Kuntz's model of protein solutions and living cells in fact maintains that the observed T_1 is determined primarily by the Type II water and the observed T_2 by the Type III water. This masking of the T_1 and T_2 of the large fraction of Type I water by the relaxations of the minor fractions of water makes it difficult to learn much about the nature of the bulk phase water in these cases from the T_1 and T_2 data. Nevertheless, Cooke and Kuntz did draw the conclusion from their NMR studies that the bulk of water in living cells is normal liquid water. This conclusion was perhaps founded partly on the similarity in the behavior of protein solutions and living cells they studied, and partly on the assumption that if the bulk phase cell water is in the state of polarized multilayers, its τ_c would be much longer than that of normal water and the T_1 and T_2 would no longer agree with the values observed. The data on the polymer-dominated water presented here leads us to conclude that this assumption is not correct.

Estimates of τ_c of Polymer-dominated Water. PVP, PEO, and PVME are all long linear chains of similar bifunctional monomeric units joined end to end. There are no polar side chains, hence no Type II water. Nor is there the basis for postulating the exis-

tence of special sites that can powerfully restrict the rotational motional freedom of water molecules as suggested for Type III water in protein solution (Cooke and Kuntz). In agreement with these simple facts, T_1 and T_2 of water in solutions of these three polymers are essentially equal over the whole range of polymer concentration studied, much as they are in distilled water. A comparison of the data with Figure 13 shows that no significant population of water protons could have τ_c much longer than that corresponding to the T_1 minimum at 3×10^{-8} sec. Further analysis, to be described later, shows that the τ_c is in fact much shorter than this.

Since sites that theoretically could produce Cooke and Kuntz's Type II and III water cannot exist in these polymers, the reduced T_1 and T_2 when compared to those of liquid water can be explained in only two ways: All water at all polymer concentrations is influenced by the polymers; this is not impossible but rather unlikely. A more likely interpretation is that some water molecules are significantly affected by the polymers and the rest of the water molecules are normal liquid water.

Even though from the viewpoint of the polarized multilayer theory, there cannot be sharp boundaries between water molecules affected by the polymers and those that are not, it is, nevertheless, convenient to assume arbitrarily that water in the PVP, PEO, and PVME solution can be divided into two kinds. They are designated as polymer-altered water (PAW) and normal liquid water. Our next questions are: (1) "What part of the polymer molecules could have affected the water?" (2) "How much water is altered by the polymer at each polymer concentration?" and finally (3) "What is the correlation time, τ_c , of this polymer-altered water?"

1. *What part of the polymer molecules alter the water?* The structures of PEO and PVME are very simple; there are only two types of atoms or atomic groups: CH_2 and

CH₃ on the one hand, oxygen on the other. There is no significant interaction between saturated hydrocarbons and water; indeed it is this lack of hydrocarbon-water interaction that creates the so-called "hydrophobic bonds." This leaves oxygen atoms as the primary seats of water-polymer interaction. There is other evidence supporting this conclusion. As an example, in his studies of amide-water interaction, Wolfenden (1978)⁵⁴ found that the peptide oxygen, not the NH group, is the major site of interaction with water.

2. *How much water is altered by the polymers at each polymer concentration?* Due to the masking effect of any rapid exchange of magnetic energy among water molecules and between water and non-water molecules or atoms by spin diffusion, it is difficult to tell from NMR data how many water molecules in a complex system like a polymer solution have altered NMR relaxation times. However, it is here that the property of solvency reduction of the water is most useful. Thus if a p -value of 0.5 for a certain probe molecule is demonstrated, at least 50% of the water must be affected. In Tables 3 and 4, we have included the p -values for Na citrate of solutions of the two polymers PEO and PVME from Ling and Ochsenfeld (1983).⁵⁵ Also cited in these tables are the estimated minimal amount of polymer-altered water given in percentage. Thus at 50% concentration, at least 90% of the water of both PEO and PVME solutions has lost its solvency for Na citrate. In 7.5% PEO solutions, only 13% of the water has been so affected while in 7.5% PVME 50% of the water has lost its solvency for Na citrate. Moreover, the altered water is not likely to have lost completely its solvency. In that case, the percentage of polymer-altered water will be even greater.

3. *What is the rotational correlation time τ_c of the polymer-altered water?* Once we know how much of the water in a particular concentration of PEO or PVME is made non-solvent to Na citrate, we can calculate the T_1 of this altered water from the measured T_1 of that polymer solution by making use of Equation 1. The fact that only a single T_1 (and T_2) was observed, justified the use of the rapid exchange model on which Equation 1 was based. For an illustrative discussion we may choose a 50% PEO solution. Here the measured T_1 taken from the data of Figure 8, and tabulated in Table 3 is 520 msec or 0.52 seconds. Since the un-degassed water measured with our spectrometer has a T_1 of 2.6 sec. (Table 1), the average T_1 of the polymer-altered water, represented as T_1^{PAW} is described by

$$\frac{0.92}{T_1^{\text{PAW}}} + \frac{0.08}{2.6} = \frac{1}{0.52} \quad (2)$$

where 0.92 and 0.08 are respectively the volume percentage of polymer-altered water and normal liquid water respectively. From this equation, a T_1^{PAW} of 487 msec. is calculated. This and other data are recorded in Table 3.

A survey of the T_1^{PAW} in Tables 3 and 4, reveals that, as a rule, its value increases as the percentage of polymer decreases. Based on the theoretical curve of Figure 13, we also estimated the rotational correlation time, τ_c , for the polymer-altered water. The τ_c values of T_1^{PAW} given in Tables 3 and 4 range from 7.6×10^{-12} second for 7.5% PVME to 4.88×10^{-11} for 50% PVME. Comparing these with the τ_c of normal liquid water (3×10^{-12} second) one finds that (i) it is longer but only by a very modest factor and (ii) the τ_c for the polymer-altered water ranges from 3 to 19 times slower than the τ_c of normal liquid water. We caution that the precise values of τ_c estimated must not be overly emphasized. First, the exact values of constants used in computing the curves are subject to some variation. Second, the theoretical curve of rotational correlation time we used refers

only to the intramolecular dipolar interaction ($T_{1\text{intra}}$) while the observed longitudinal relaxation (T_1) originates from both $T_{1\text{intra}}$ and intermolecular interaction ($T_{1\text{inter}}$), involving largely diffusional motion. Estimates of from 25% (Abragam, 1961, p. 302) to as high as 50% (Emsley, Feeney, and Sutcliffe, 1965; Krynicki, 1966) of the T_1 of water proton may originate from the intramolecular effect. Since

$$T_1^{-1} = T_{1\text{intra}}^{-1} + T_{1\text{inter}}^{-1}, \quad (3)$$

by ignoring the intermolecular contribution, we in fact somewhat overestimate the value τ_c . But this error is not very significant in the degree of quantitative accuracy we strive for now.

A New Interpretation of the NMR Data from Living Cells. Up to now, major disagreements exist on the subject of the physical state of water in living cells. From NMR studies some investigators claim the bulk of cell water is normal. Others objected to this view because of the insufficiency of energy to operate the membrane-pumps which the normalcy of cell water inevitably demands (for full discussion on this subject, see Ling, 1962, 1983).

As mentioned earlier, part of the free-cell-water conclusion might have originated from an erroneous guess of what τ_c should be for water protons in the state of polarized multilayers. The experimental demonstration presented here shows that the τ_c of water fully able to exclude Na citrate may, nevertheless, be different from that of normal water by a modest factor of 3. This modest lengthening of τ_c opens the door toward a new interpretation of the NMR data of water in living cells.

First we must understand that the foundation for the conclusion of normal liquid cell water is of limited precision and involves assumptions that seemed reasonable but not proven. Thus Cooke and Kuntz estimated

Type II water to range from 0.3 g. protein to twice that much. They also considered that Type II water is the non-freezable water. Kuntz et al (1969) showed that the non-freezable water in a BSA solution amounts to 0.4 g. H_2O g. protein. It has long been established that 60% gelatin does not freeze even in liquid nitrogen (Moran, 1926). Thus the non-freezable water of gelatin is $40/60 = 0.67$ g. H_2O g. protein. Comparing the two, one concludes that a gelatin solution has much more Type II water than one of BSA of equal concentration. With more Type II water, the T_1 of a gelatin solution should be considerably shorter than that of an equal concentration of BSA.

Now Cooke and Kuntz estimated the "intrinsic" T_1 for Type II water at 40 msec, a value corresponding to a τ_c of 10^{-9} sec. At this τ_c value, T_1 is independent of the field frequency (see Figure 13). Thus their data of 40 msec or .04 sec. estimated at 100 MHz can be directly applied to our measurements at 17.1 MHz. Using Equation 1 we have for a 20% solution of BSA and a value of 2.6 second for T_1 of normal liquid water, a predicted T_1 for water protons given by:

$$\frac{1}{T_1} = \frac{1-(0.2 \times 0.4)}{2.6} + \frac{0.2 \times 0.4}{0.04}, \quad (4)$$

$$T_1 = 426 \text{ msec.} \quad (5)$$

For a 20% gelatin solution,

$$\frac{1}{T_1} = \frac{1-(0.2 \times 0.66)}{2.6} + \frac{0.2 \times 0.66}{0.04}, \quad (6)$$

$$T_1 = 275 \text{ msec.} \quad (7)$$

Now let us compare these theoretical values with those actually measured. An examination of Figure 3 shows that T_1 of 20% BSA is 620 msec, which is not a good fit of the predicted value of 426 msec but perhaps acceptable. A much worse prediction is that of the gelatin solution. Thus T_1 of a 20% gelatin solution actually measured is not shorter than that of BSA as predicted.

Rather it is longer considerably, at 1300 msec!

This rather severe discrepancy between theoretical predictions and experimental observations illustrates first that the non-freezable water may not necessarily be the source of T_1 reduction in all protein solutions. Second, we suspect that non-freezable water may not be always equated with hydration water of polar side chains (Bull and Breese, 1968; Kuntz et al, 1969). Were it otherwise, there should be no non-freezing water in solutions of PEO, PVME, and PVP at all. None of these polymers contains polar side chains but they all show large amounts of non-freezing water as will be described in detail in a forth-coming paper (Zhang and Ling, 1983).

From the data of Ling and Ochsenfeld (1983), one finds that the ρ -value for Na citrate in a 20% gelatin solution is 0.87. If one assumes that there are only two kinds of water in this gelatin solution, "non-solvent" water and normal liquid water, they would correspond, respectively, to 13% and 87% of the total water. Again using Equation 1, and the measured T_1 of 1300 msec for a 20% gelatin solution one finds that

$$\frac{1}{1.3} = \frac{0.87}{2.6} + \frac{0.13}{T_1^{PAW}} \quad (8)$$

and

$$T_1^{PAW} = 0.300 \text{ second.} \quad (9)$$

A comparison with the T_1 of non-solvent water in the PEO and PVME systems at similar concentrations given in Tables 3 and 4 suggests that 300 msec may be too low a figure to be consistent with the PEO and PVME data. That is, in promoting water proton relaxation, gelatin may have in addition to what it shares with PEO and PVME, something extra. What can this extra element be? Probably polar side chains.

So we have come a full cycle back to the polar side chains as a major source of water

proton NMR relaxation in proteins. Indeed, another compelling reason for its involvement is the even shorter T_1 for native BSA solution, which Ling et al (1980a, b) have shown to contain little "non-solvent water" for Na sulfate. However the role of polar side chains in causing water proton relaxation may be more complicated than that of a direct equation with hydration water of polar side chains. Some specific polar side chains or perhaps combinations of polar side chains of the same or even neighboring protein molecules may be more effective than others.

If we assume that water influenced by the "polar side chains" or "polar-side-chain complexes" has a T_1 of about 40 msec, one can also make a rough estimate as to how much of this water (x) exists in a 20% gelatin solution, assuming that the non-solvent water has the same T_1 as in PEO or PVME solutions at an equal concentration.

$$\frac{1}{1.3} = \frac{(1-0.13-x)}{2.6} + \frac{0.13}{1.2} + \frac{x}{0.04} \quad (10)$$

$$x = 0.013. \quad (11)$$

Thus only 1.3% of the water in the 20% gelatin needs to be influenced by the "polar side chains" in order to produce a T_1 of 1300 msec in a 20% gelatin solution.

Now let us turn to living cells. From the AI Hypothesis, the bulk of cell water would be non-solvent to Na citrate; none or very little free water exists in normal resting cells. Ling also expressed the opinion that paramagnetic ions associated with cell proteins most likely contribute to the water proton relaxation (Ling, 1979a; Ling and Tucker, 1980). To discuss NMR properties of water protons in living cells some specific data are needed. We cite the following: On December 26, 1980, four pairs of sartorius muscles from four male frogs were isolated. Using the same instruments the T_1 measured was, for each pair: 580, 600; 610, 610; 680, 690; 620, 640 msec. The mean \pm S.D. was 629 ± 38.7 msec. The water contents were all close to 80%.

Let us lump the fast-relaxing water associated with paramagnetic ion-protein complexes and those associated with special "polar side chains" of cell proteins and represent their sum as y with a T_1 equal to .04 sec. Again assuming the bulk of cell water to have the same T_1 as in a 20% PEO or PVME solution (1.3 sec.), we have

$$\frac{1}{0.63} = \frac{0.8-y}{1.3} + \frac{y}{0.04} \quad (12)$$

$$y = 0.04. \quad (13)$$

Thus only 4% of the muscle cell water needs to be associated with paramagnetic ion-protein complex and with special "polar-side chains" to account for the observed T_1 in frog sartorius muscle while the bulk of cell water is "non-solvent." This single set of figures has shown that it is possible to explain at once the NMR data and the low levels of Na^+ , sugar, free amino acids, etc., in living cells, without postulating an ever-lengthening list of membrane pumps.

We now turn to another aspect of the findings described in this paper, i.e., the variability of τ_c of the "non-solvent" water and the increase of τ_c with decreasing water content and increasing polymer concentration. We shall demonstrate that this facet of the present finding may help to clarify some other current conflicts of opinions.

The Increase of Motional Restriction in the "Non-solvent" Water with Increasing Polymer Concentration as the Basis for Reconciling Seemingly Contradictory Findings of the Dielectric Properties of Cell Water. Studies of cells of *Artemia* cysts revealed a dielectric constant of the cell water distinctly different from that of normal liquid water (Clegg et al., 1982).³² In contrast, Foster, Schepps, and Schwann (1980)³³ failed to find significant differences in the dielectric constant of water in isolated barnacle muscle at 0°C when compared to normal liquid water.

First we would like to mention that in our experience with barnacle muscles (Reisin and Ling, 1973),³⁴ the isolation of the muscle cells from the barnacle shell involves inevitable cutting and severe damage of one end of these muscles. This deleterious effect, which tends to spread, seems exacerbated by subsequent exposure to 0°C. It would seem that a better alternative is to study frog muscles, which come in intact form and are entirely healthy at 0°C. However, even if frog muscles were studied, it would not be altogether surprising that the data do not completely agree with those from the study of *Artemia* cysts for the following reason.

We have already presented some of the reasons why the bulk of water in all types of living cells would be "non-solvent" to $\text{Na}_2\text{citrate}$ (for full account, see Ling, 1983b).³² Thus if the trend of decreasing τ_c with increasing water content and decreasing polymer (or protein) content (Tables 3 and 4) holds in living cells, then the much higher water contents of barnacle or frog muscle (80%) will predict a lower τ_c for muscle water protons than for those of *Artemia* cysts, which had a water content of 50%. Since the Debye rotational correlation time τ_d is equal to 2.5 τ_c , (Eisenberg and Kauzmann, 1967)³⁵ the anticipated smaller difference in τ_d of normal liquid water may be easily overlooked.

It seems fitting to end this discussion by pointing out that the recent quasi-elastic neutron diffraction studies of *Artemia* cysts also led to the conclusion that the rotational motional freedom of water in these cells was clearly restricted (Trantham et al., 1983).³⁶ Rorschach (1983)³⁷ also studied the neutron diffraction of a 35% solution of PEO, which was in fact a sample from the same PEO used in the present NMR study. He found that the PEO solution strongly resembles the *Artemia* cyst. From Table 3 one finds that at 35% PEO the estimated τ_c should be about 1.60×10^{-11} sec.

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FIGURE 1. Plots of $\ln(A_0/A)$ against τ . A_0 is the initial amplitude of the free induction decay signal following the 90° pulse at time τ . A_0 is A as $\tau \rightarrow \infty$. The data illustrate that the plots can be fitted with a single slope, equal to $-(T_1)^{-1}$. The concentrations were: BSA (10.0%); gelatin (2.94%); PVP (5.66%); PEO (7.67%); PVME (8.67%).

FIGURE 2. Carr-Purcell plots of $\ln A_t$ against t where A_t is the echo height at time t . The data illustrate that the plots can be fitted with a single slope equal to $-(T_2)^{-1}$. The concentrations were: BSA (8.3%); gelatin (8.0%); PVP (9.3%); PEO (6.1%); PVME (9.9%).

FIGURE 3. T_1 and T_2 of water proton in aqueous solutions of bovine serum albumin plotted against their water contents.

FIGURE 4. T_1 and T_2 of water proton in aqueous gelatin gel plotted against their water contents.

FIGURE 5. Plots of $(T_1)^{-1}$ (●) and $(T_2)^{-1}$ (○) against the water contents of different concentrations of bovine serum albumin (BSA). Data same as in Figure 3.

FIGURE 6. Plots of $(T_1)^{-1}$ (●) and $(T_2)^{-1}$ (○) against the water contents of different concentrations of gelatin gel. Data same as in Figure 4.

FIGURE 7. T_1 and T_2 water protons in aqueous solutions of polyvinylpyrrolidone (PVP) against their water contents.

FIGURE 8. T_1 and T_2 of water proton in aqueous solutions of poly(ethylene oxide) (PEO) against their water contents.

FIGURE 9. T_1 and T_2 of water protons in aqueous solutions of polyvinylmethylether (PVME) against their water contents.

FIGURE 10. Plots of $(T_1)^{-1}$ (●) and $(T_2)^{-1}$ (○) against the water contents of the solutions of

FIGURE 10. Plots of $(T_1)^{-1}$ (●) and $(T_2)^{-1}$ (○) against the water contents of the solutions of polyvinylpyrrolidone of different concentrations. Data same as Figure 7.

FIGURE 11. Plots of $(T_1)^{-1}$ (●) and $(T_2)^{-1}$ (○) against the water contents of poly(ethylene oxide). Data same as in Figure 8.

FIGURE 12. Plots of $(T_1)^{-1}$ (●) and $(T_2)^{-1}$ (○) against the water contents of various polyvinylmethylether solutions. Data same as in Figure 9.

FIGURE 13 Theoretical curves of T_1 and T_2 of water proton at various rotational correlation times, τ_c , calculated from the equations of Bloembergen, Purcell and Pound (1948) as modified by Kubo and Tomita (1959)

$$(T_{1\text{ intra}})^{-1} = \frac{3\gamma^4 h^2}{10b^3} \left[\frac{\tau_c}{1 + \omega_0^2 \tau_c^2} - \frac{4\tau_c}{1 + 4\omega_0^2 \tau_c^2} \right] \text{ and}$$

$$(T_{2\text{ intra}})^{-1} = \frac{3\gamma^4 h^2}{20b^3} \left[3\tau_c - \frac{5\tau_c}{1 + \omega_0^2 \tau_c^2} - \frac{2\tau_c}{1 + 4\omega_0^2 \tau_c^2} \right]$$

where γ is the magnetogyric ratio (equal to 5585 for protons), h is the Plank constant ($h/2\pi$), b is the interproton distance, ω_0 is the Larmour frequency (17.1×10^6 Hz for our instrument). The value of $A =$

$\frac{3\gamma^4 h^2}{20b^3}$ used was $0.83 \times 10^{-4} \text{ s}^{-2}$

TABLE 1. T_1 and T_2 of water protons of distilled water at different pH's in air or nitrogen.

Distilled Water	pH	T_1 (msec)	T_2 (msec)	T_1/T_2
Air	6.6	2550	1740	1.47
N ₂	6.6	3200	2100	1.52
Air	3.5	2550	2150	1.21
N ₂	3.5	3250	2700	1.20
Air	10.6	2600	2200	1.30
N ₂	10.6	3325	2650	1.28

TABLE 2. The ratios of T_1/T_2 for BSA, gelatin, PVP, PEO, and PVME at various water contents. To save space, every 4th set of data from PVP and every second set of data from PEO were deleted. These deleted data are in essence similar to those retained.

BSA		Gelatin		PVP		PEO		PVME	
% H ₂ O	T_1/T_2	% H ₂ O	T_1/T_2	% H ₂ O	T_1/T_2	% H ₂ O	T_1/T_2	% H ₂ O	T_1/T_2
98.0	1.49	89.5	1.63	90.7	1.10	95.9	1.37	91.2	1.32
91.7	2.27	74.9	2.54	81.6	1.41	90.8	1.41	83.0	1.30
86.5	2.25	59.3	4.67	76.4	1.27	87.4	1.54	75.4	1.27
79.2	3.30	47.2	9.29	69.9	1.06	77.1	1.40	65.9	1.10
70.0	5.27	42.4	5.50	67.1	1.28	77.8	1.39	59.4	1.11
59.0	11.50	35.6	12.50	59.9	0.98	66.4	1.45	40.4	1.01
		24.8	13.00	41.8	1.20	56.3	1.20	26.8	1.82
				39.0	0.74	39.9	1.20		

TABLE 3. The T_1^{PAW} and τ_c^{PAW} of estimated polymer altered water (PAW) in PEO-water system. The amounts of PAW were obtained from the p -values for Na citrate and were taken from Ling and Ochsenfeld (1983). Method of calculations described in text.

PEO							
Polymer Content (%)	7.5	10	15	20	30	40	50
H ₂ O Content (%)	92.5	90	85	80	70	60	50
T_1 (msec)	2280	2110	1800	1540	1110	780	520
p -value	0.87	0.73	0.51	0.39	0.22	0.13	0.08
Minimal polymer altered water (%)	13	27	49	61	78	87	92
T_1^{PAW} (msec)	1250	1400	1360	1220	947	707	487
τ_c^{PAW} (sec)	1.02×10^{-11}	9.2×10^{-12}	9.4×10^{-12}	1.06×10^{-11}	1.36×10^{-11}	1.81×10^{-11}	2.67×10^{-11}

TABLE 4. The T_1^{PAW} and τ_c^{PAW} of estimated polymer altered water (PAW) in PVME-water system. The amounts of PAW were obtained from the p -values for Na citrate and were taken from Ling and Ochsenfeld (1983). Methods of calculations described in the text.

PVME					
Polymer content (%)	5	7.5	10	15	20
H ₂ O content (%)	95	92.5	90	85	80
T_1 (msec)	2100	2050	1950	1750	1540
p -value	0.89	0.50	0.45	0.39	0.34
Minimal polymer altered water (%)	11	50	55	61	66
T_1^{PAW} (msec)	1821	1690	1620	1450	1270
τ_c^{PAW} (sec)	1.55×10^{-11}	7.6×10^{-12}	7.9×10^{-12}	8.9×10^{-12}	1.03×10^{-11}

30	40	50	60
70	60	50	40
1130	730	480	340
0.22	0.11	0.05	0.12
78	89	95	88
975	670	461	268
1.33×10^{-11}	1.93×10^{-11}	2.80×10^{-11}	4.80×10^{-11}

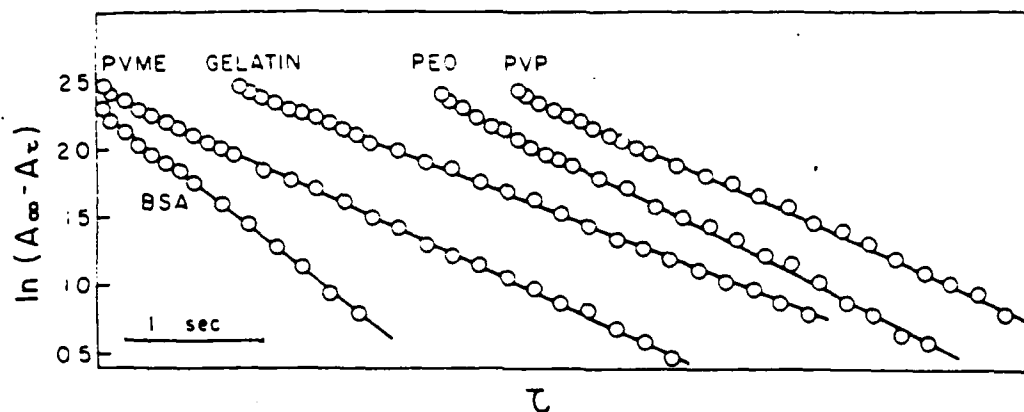


FIGURE 1

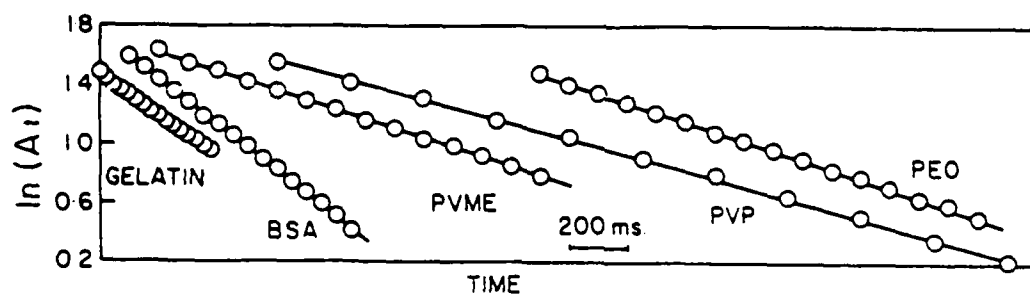


FIGURE 2

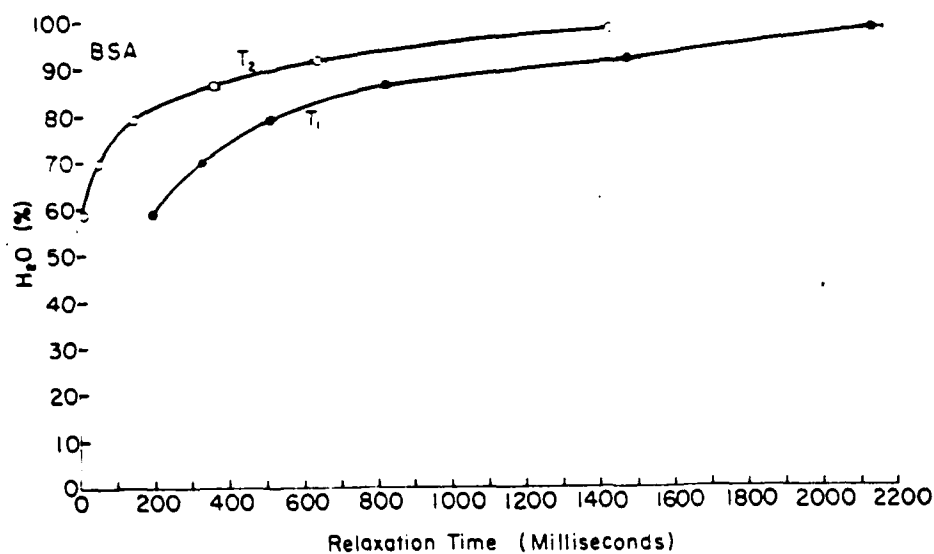


FIGURE 3

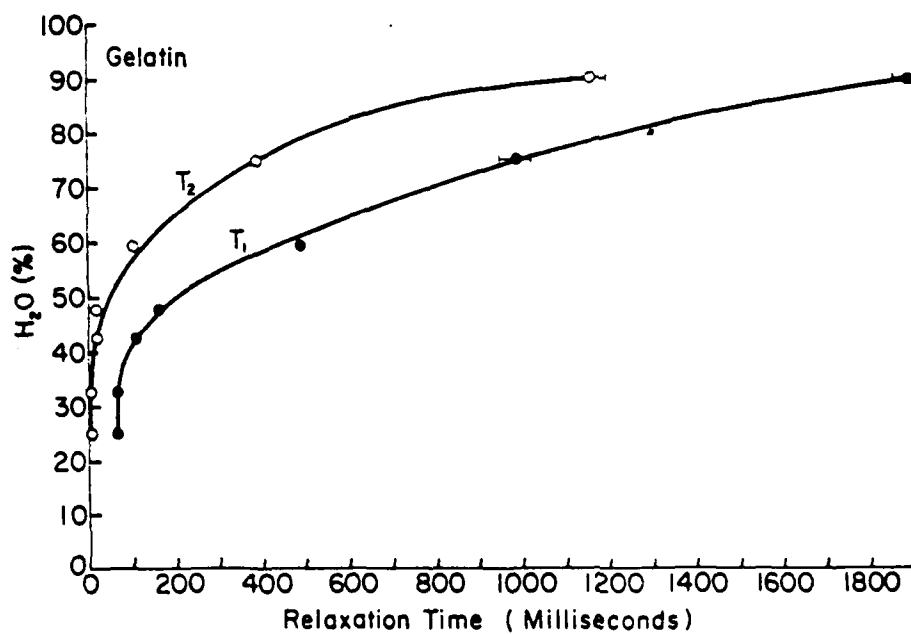


FIGURE 4

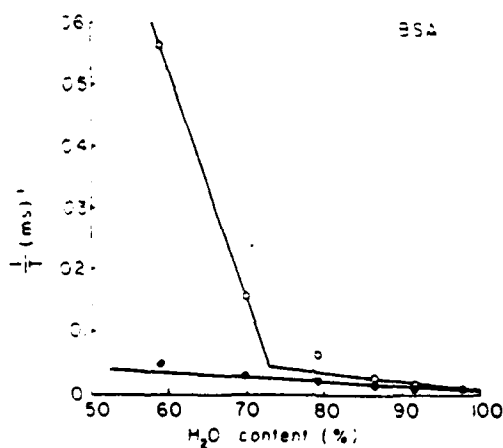


FIGURE 5

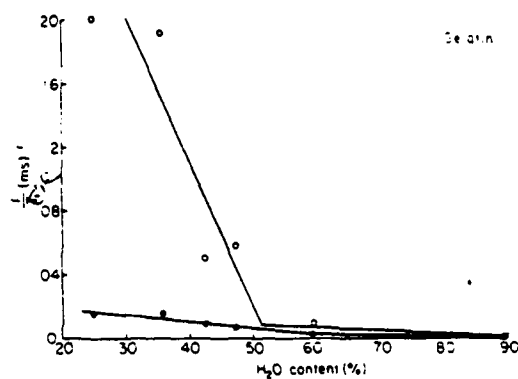


FIGURE 6

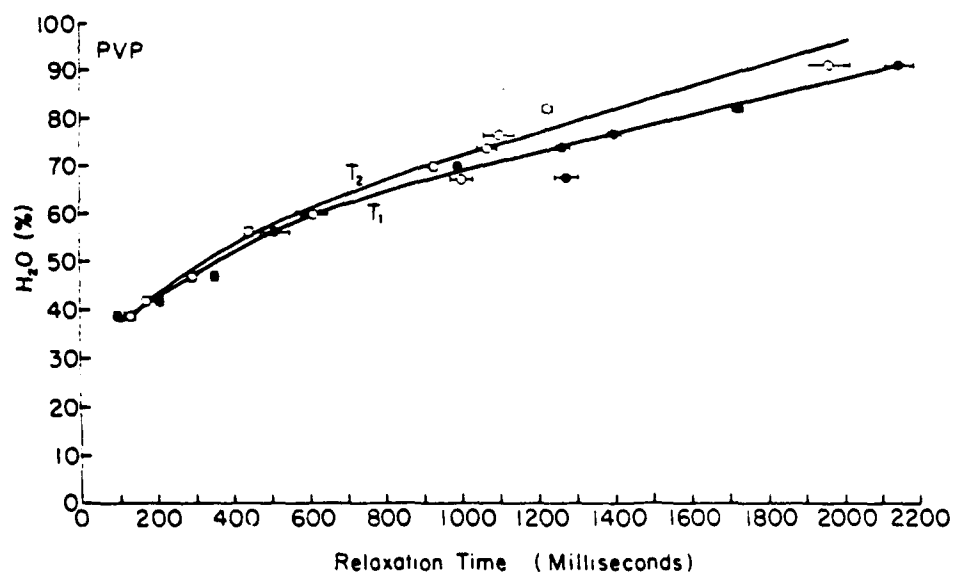


FIGURE 7

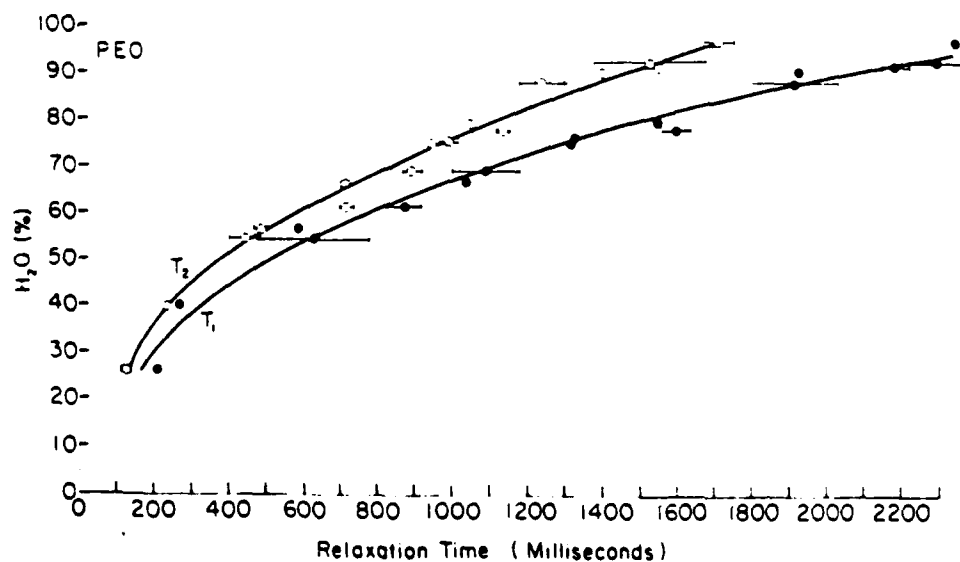


FIGURE 8

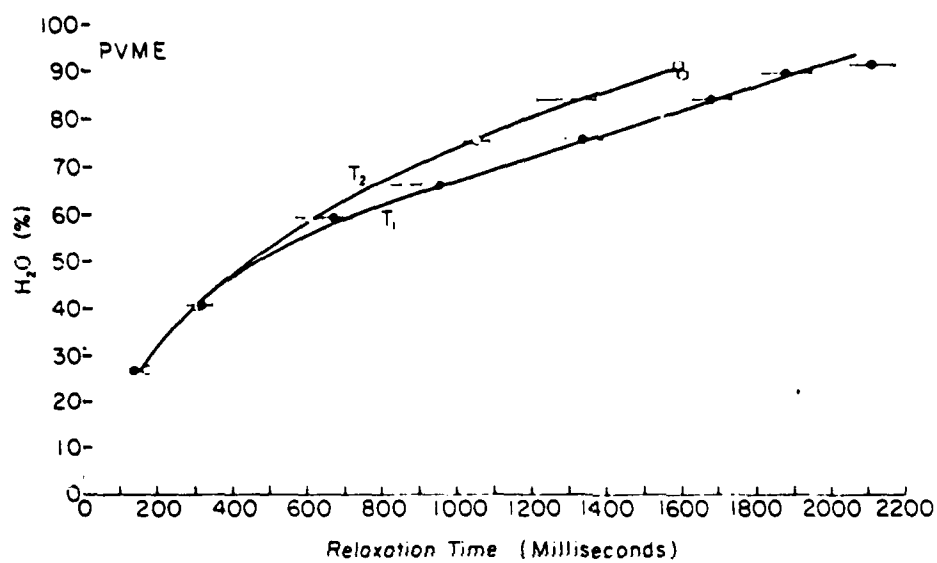


FIGURE 9

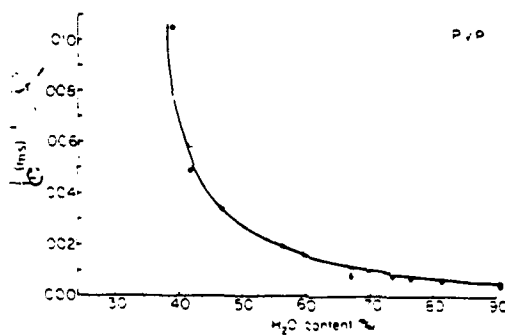


FIGURE 10

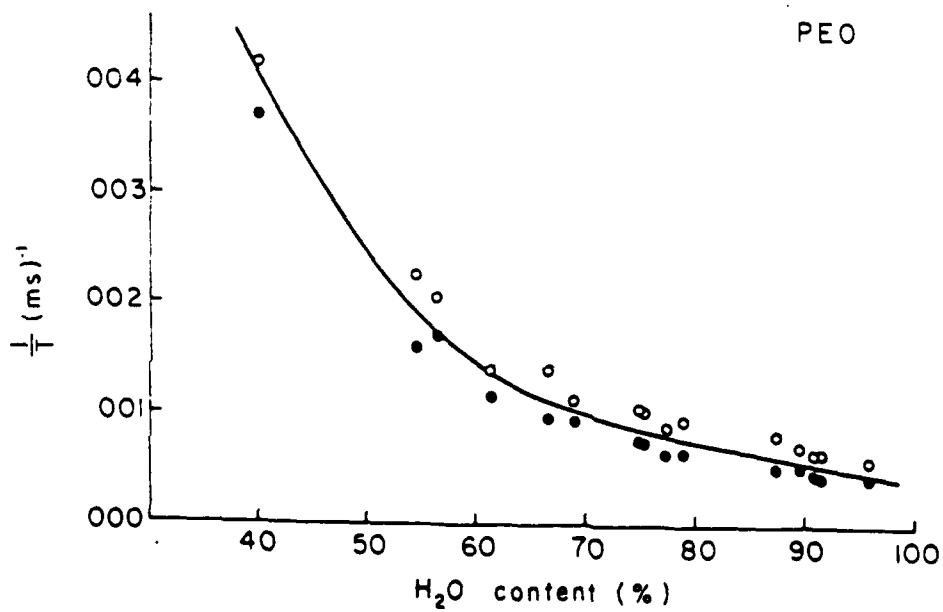


FIGURE 11

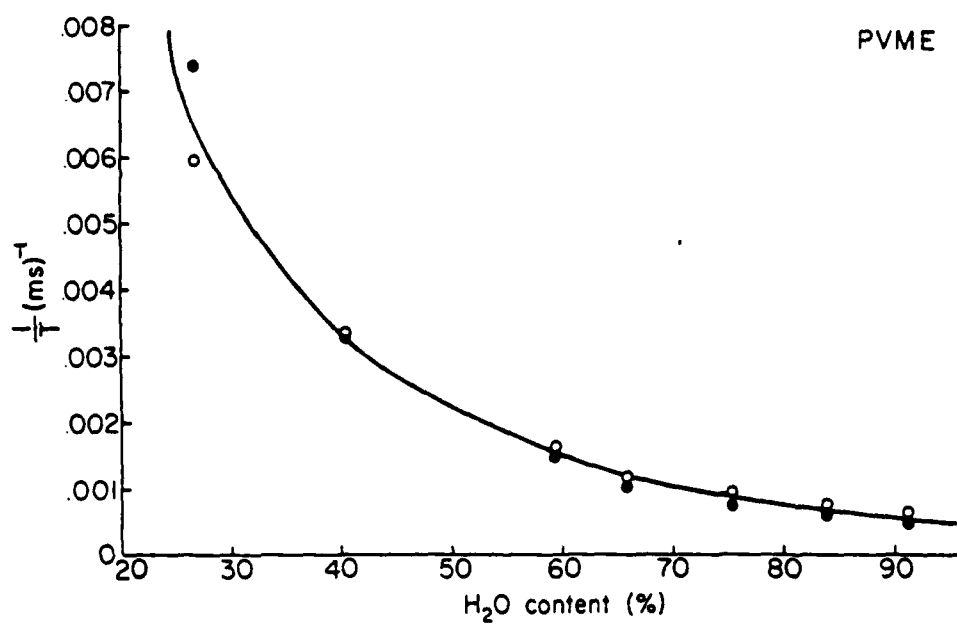


FIGURE 12

NMR RELAXATION OF WATER PROTONS UNDER THE INFLUENCE OF PROTEINS AND OTHER LINEAR POLYMERS

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The concept that water in living cells may exist in a physical state different from that of normal liquid water¹⁻⁵ stimulated considerable interest in the nuclear magnetic resonance (NMR) relaxation times of water protons in living cells,⁶⁻⁸ (for review, see ref. 9). The low T_1 and T_2 , as well as the high ratio of T_1/T_2 observed in living cells, led some scientists to the belief that the NMR data lent direct support to the association-induction hypothesis, according to which all or nearly all cell water exists in a state of polarized multilayers (PMW).

Subsequent investigations, however, led many to a different conclusion. It was argued that the short T_1 , T_2 and high T_1/T_2 ratio reflect only the properties of a minor-phase water in rapid exchange with bulk-phase water, which is simply normal liquid water.⁹⁻¹⁰

It is gratifying to note that these efforts aimed primarily at solving a basic science problem have already led to the recognition of one fundamental attribute of cancer¹¹ and the development of a potentially powerful tool for clinical medicine, the FONAR.¹² In both, Damadian played a major and critical role.

A major difficulty in using NMR relaxation time studies to determine whether or not the bulk-phase water exists in the PMW state lay in the lack of recognized criteria by means of which NMR proton relaxation times could be used to differentiate normal water and PMW. This lack of known distinguishing NMR traits of PMW in turn reflected the fact that at the time the PMW model was proposed there was no readily available

specimen of water firmly established as existing in the polarized multilayer state. Without an inanimate model, the assumption that the major phase cell water is entirely normal liquid water became the more appealing, whereas in truth the "cover-up" effect of a minor phase rapidly relaxing water is so powerful that the bulk-phase water may very well have relaxation times quite different from those of normal liquid water and still remain camouflaged.

Over the last few years, however, this laboratory has succeeded in developing a simple but efficient method of diagnosing PMW. By use of that method, the existence of PMW has been established as well as the conditions that convert normal liquid water into PMW. Specifically, the method consists of exposing water to a matrix of fairly closely placed chains containing oxygen atoms, the distances between the nearest neighboring oxygen atoms being roughly equal to twice the diameter of a water molecule.^{13,14} The number of layers of water that can be effectively polarized between chains is estimated as falling below 10 molecules between a pair of polarizing chains.

Among the water-affecting polymers studied as matrices, the most intriguing is poly (ethylene oxide) (PEO) $(-\text{CH}_2-\text{O}-\text{CH}_2)_n$ because of its extreme simplicity; this polymer has no side chains whatsoever. Other effective polymers are polyvinylmethyl ether (PVME), polyvinyl-pyrrolidone (PVP), and gelatin.

We have now carried out a series of studies of the NMR proton relaxation times of water in highly purified polymer-water systems.

Reserving the detailed data for a full presentation elsewhere, we report here the following basic findings:

(1) Both T_1 and T_2 of the polymer-oriented water are considerably shorter than those of normal liquid water, becoming shorter with increase of polymer concentrations.

(2) The ratio T_1/T_2 , on the other hand, is close to unity (1.0 to 1.4) in all concentrations of PEO, PVME, and PVP solutions; it resembles the T_1/T_2 ratio of normal liquid water at near neutral pH.¹⁵

(3) Gelatin solution like that of native bovine serum albumin, shows a much higher T_1/T_2 ratio at high polymer concentrations.

Taken as a whole, the data can be interpreted as follows: water in the dynamic state of polarized multilayers does indeed suffer rotational (and translational) motional restriction¹⁻⁵ but far less than that seen in solid ice. The correlation time, τ_c , for water effectively excluding Na^+ , sucrose, and glycine, is estimated to be no larger than 3.5×10^{-11} sec (25°C) and thus not more than 10 times slower than that in normal water. τ^c progressively decreases with decreasing water content of the polymer-water system, reaching a value of 10^{-11} sec at 20 to 35% water contents. Since the Debye dielectric rotational correlation time (τ_{rd}) is equal to $2.5 \tau_c$,^{16,18} the corresponding τ_{rd} should be 2.5×10^{-11} sec. It is interesting to compare this value with the additional dispersion of wet lysozyme powder with a τ_{rd} of 2×10^{-11} sec that Harvey and Hoekstra¹⁹ observed when the water content increased beyond 0.35 g/g protein. This water content corresponds roughly to the limit of polar group hydration.²⁰ Therefore the additional hydration referred to might be largely due to polarization by the polypeptide NHCO groups and thus would be polarized in a manner similar to polarization of the PEO-water system.^{13,14,20}

The T_1/T_2 ratios were found to be quite different in the two proteins studied. Here a minor phase of more rapidly relaxing water in rapid exchange with the major phase water

can explain the different behavior of both native bovine serum albumin solution, whose water is essentially normal as judged by its solvency for Na^+ , sucrose, and glycine, and of gelatin "gel" where water has reduced solvency for these probe molecules.¹³ □

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PRELIMINARY NOTE
APPARENT SIMILARITY IN PROTEIN COMPOSITIONS OF MAXIMALLY
DEVIATED CANCER CELLS

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Using SDS gel electrophoresis, we examined the total protein contents of 14 types of mouse cancer cells (Kreb's, Reif-Allen, P815, Hepatoma 134, P4132, LSA, TA3, L1210, P1081, Ehrlich, Meth. A, 15091A, Sarcoma 180, T241) and 5 types of rat cancer cells (Walker 256, Yoshida hepatoma, Novikoff, AS30, Dunning leukemia). We then compared those contents with the cellular protein contents of normal mouse and rat tissues (brain, muscle, liver, spleen, heart, lung, nerve).

The results show, on the one hand, much similarity in the kinds and amounts of proteins from the various types of cancer cells although they derived originally from widely different tissues. On the other hand, great diversity is seen among the proteins from normal cells, as to be expected. Eight of the major polypeptide bands seen in all cancer cells studied gave apparent molecular weights of 34,000, 36,900, 46,100, 49,800, 57,000, 59,200, 69,600 and 92,500 daltons respectively. All the cancer cells were what Potter¹ calls "maximally deviated" as indicated by their very short transplantation time (i.e., one week).

Our findings, to be fully described elsewhere, extend and are in harmony with the conclusions of J. Greenstein^{2,3} from his studies of one special kind of protein, the enzymes. These he found different in normal tissues but more alike in the cancer cells he studied.

The present results suggest that cancer may indeed represent a cellular change to either a single ontologically earlier totipotent state or to a single new totipotent state. In either case, apparently actively transcribed genes specific to their parent normal tissues are shut off and a specific assembly of genes common to all cancer cells is transcribed to produce highly similar if not identical cancer cells regardless of their ancestry. □

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83 12 21 001

THE RESTING POTENTIAL OF FROG MUSCLE CELL IS INDIFFERENT TO EXTERNAL Mg^{++}
EVEN THOUGH Mg^{++} IS SUBSTANTIALLY MORE PERMEANT THAN K^{+}

by

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Running Title: Mg^{++} on Muscle Resting Potential

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83 12 21 017

SUMMARY

At constant external K^+ and Na^+ concentration, variation of external Mg^{++} concentration between 1.2 mM to 73.2 mM brought about no change of the resting potential of frog sartorius muscles which remained constant at 88 mV even though Mg^+ (as chloride and sulfate) penetrates into muscle cells faster than K^+ (as chloride). The significance of these findings in relation to current theories of cellular potentials were discussed.

INTRODUCTION

A number of theories exist concerning cellular electrical potential, among which are the membrane potential theory of Hodgkin and Katz, first presented in 1949⁽¹⁾, and the surface adsorption potential theory, a part of the association-induction hypothesis presented first briefly in 1955⁽²⁾ and later in greater detail in 1960, 1962, 1982, and 1983⁽³⁻⁷⁾. Considerable experimental work has already been reported in the literature aimed at testing the validity of both these theories and will be considered later in this paper, under Discussion. This paper will present the results of yet another investigation designed to provide additional factual guidelines for determining which theory is the more realistic one.

THEORY

I. The Membrane Pump Theory of Hodgkin and Katz

Details of this well-known theory will not be reiterated here. Briefly, this theory of cellular potential is seen as a modification of the membrane potential theory which was first clearly defined by Bernstein⁽⁸⁾. In Bernstein's original version of the theory, cellular potential is described as an equilibrium potential; its maintenance per se does not demand continuous energy expenditure. The discovery that Na^+ , long thought to be an impermeant cation, is actually a permeant cation^(9,10) renders this version of the membrane theory untenable. Partly to overcome this difficulty and partly to incorporate their important discovery of the key role of Na^+ in the creation of the action potential⁽¹¹⁾, Hodgkin and Katz presented their own conception of cellular potential, which though still retaining the basic characteristics of the membrane potential was no longer an equilibrium potential⁽¹⁾; maintenance of this potential now depended on the continuous operation of an energy-consuming pump^(12,13).

The Hodgkin-Katz model of cellular potential is described by the following equation:

$$\psi = \frac{RT}{F} \ln \frac{P_K [K^+]_{in} + P_{Na} [Na^+]_{in} + P_{Cl} [Cl^-]_{ex}}{P_K [K^+]_{ex} + P_{Na} [Na^+]_{ex} + P_{Cl} [Cl^-]_{in}}, \quad (1)$$

where P_K , P_{Na} , and P_{Cl} are the membrane permeability constants of the respective ions; $[K^+]_{in}$, $[Na^+]_{in}$, and $[Cl^-]_{in}$ are their intracellular concentrations; and $[K^+]_{ex}$, $[Na^+]_{ex}$, and $[Cl^-]_{ex}$ are their extracellular concentrations ⁽¹⁾.

R , F , and T are the gas constant, the Faraday constant, and the absolute temperature, respectively.

The basic tenet of the membrane theory demands that all particles carrying net electrical charges and capable of traversing the cell membrane contribute to the magnitude and polarity of the electrical potential. Thus, ideally, the equation for cellular potential should incorporate all permeant ions found inside and outside the cells under the condition when the potential is measured. The Hodgkin-Katz equation, however, deals explicitly with only three ions: K^+ , Na^+ , and Cl^- . The ubiquitous divalent Mg^{++} and Ca^{++} cations were not included in this equation. Presumably, this omission was justified on the grounds that the intracellular as well as extracellular concentrations of Mg^{++} and Ca^{++} were lower than those of K^+ , Na^+ , and Cl^- and on the assumption that these alkaline earth ions were also less permeable than K^+ , Na^+ , and Cl^- . However, unequivocal evidence that the divalent ions have a much lower permeability is not available; indeed, evidence exists showing just the opposite. Thus Tasaki, Teorell, and Spyropoulos demonstrated that in perfused squid axons the time constant of Ca^{++} efflux was only 25 minutes while that of K^+ efflux was 8 hours ⁽¹⁴⁾. In other words, the membrane

permeability is many times higher for Ca^{++} than for K^+ ! On the other hand, the earlier conclusion that Mg^{++} permeability is very low ⁽¹⁵⁾ was challenged. Thus Ling et al ⁽¹⁶⁾ showed that the gain of external Mg^{++} in a high Mg^{++} -Ringer solution in frog muscle was complete within 20 min. even though some of the muscles used were as thick as 2mm in diameters.

In order to test the Hodgkin-Katz model, unequivocal knowledge about Mg^{++} permeability is essential. Since similar data do not appear to be available for Mg^{++} , another purpose of the present investigation was to acquire such information. If Mg^{++} should prove to be equally permeable as, for example, K^+ , the membrane potential theory would predict a substantial lowering of the resting potential or even reversal of the sign of the potential when there is a large increase in external Mg^{++} concentration.

II. The Adsorption Potential Theory According to the Association-Induction Hypothesis

According to the association-induction hypothesis, the bulk of intracellular K^+ is adsorbed on the δ - and γ -carboxyl groups of intracellular proteins ^(4,7,17) and is not involved in the generation of the electrical potentials. Instead, the potential is determined by the δ - and γ -carboxyl groups found on a microscopically thin layer of cell surface and by their preferentially adsorbed ions ^(2-4,6,7).

A. The Resting Potential

In this theory, the cellular resting potential is again an equilibrium potential, the maintenance of which does not demand a continuous expenditure of energy. Written in the simplest form possible, the cellular electrical

potential ψ is described by the equation:

$$\psi = \frac{RT}{F} \ln [f^-] - \frac{RT}{F} \ln \left\{ \tilde{K}_K [K^+]_{\text{ex}} + \tilde{K}_{Na} [Na^+]_{\text{ex}} \right\}, \quad (2)$$

where $[f^-]$ is the concentration of fixed anions on the microscopic layer of the cell surface and \tilde{K}_K and \tilde{K}_{Na} are the adsorption constants for K^+ and Na^+ , respectively, on the β - and γ -carboxyl groups on the cell surface (2-4). As mentioned earlier, this equation was first presented in 1959 and again in 1960 and 1962. A further theoretical development of this model was given in 1979 (18), its experimental support presented in 1982 and 1983 (6,7,19).

B. Characteristics and Predictions.

1. There is no causal relationship between ionic permeabilities and cellular electrical potential.

2. No macroscopic interface separating the membrane from the cytoplasm has been recognized. Instead, only one discrete interface that separates the cell surface from the surrounding medium is considered to exist. However, this is not to say that another artificial interface may not be created, such as by the removal of cytoplasm; in this case, no or little potential difference at this artificial interface is expected if the exposed surface is amphoteric, containing roughly equivalent concentrations of fixed cations and fixed anions and if the pH is near the isoionic point (6,7).

3. The major determinants in the potential are the nature and density of fixed anionic sites on the cell surface and the concentrations of ions in the surrounding medium that can adsorb to these fixed anionic sites.

Experimental evidence exists showing that the predominant fixed charges on the surface of frog muscle cells are indeed the β - and γ -carboxyl groups carried by the aspartic and glutamic residues of proteins:

a. The K^+ adsorbing sites of the muscle cell surface have a pK value of 4.6 which is characteristic of the β - and γ -carboxyl groups (20).

b. The cell surface binds cationic uranium ions in EM sections of fixed cells. Recently, with the aid of transmission electron microscopy (21) and of autoradiography (22,23), dispersive x-ray microprobe analysis (24-26), and laser mass spectrometer microprobe analysis (LAMMA) (27), evidence have been presented suggesting that cytoplasm K^+ adsorption sites are the β - and γ -carboxyl groups which in fixed cell preparations may also bind uranium ions (see below).

There is reason to believe that ionic preference in adsorption varies not only with respect to the nature of the fixed anionic groups, their c-value (see ref. 4), but also with respect to their spatial distribution in relation to other fixed anions. Thus, isolated fixed carboxyl groups seem to prefer alkali-metal ions (Cs^+ , Rb^+ , K^+ , Na^+ , Li^+) over the divalent ions Mg^{++} , Ca^{++} , and Sr^{++} . As an example, oxidized collodion possessing a relatively low concentration of carboxyl groups (28) shows little or no tendency to adsorb alkaline earth ions; it does adsorb alkaline metal ions avidly (29).

On the other hand, when fixed carboxyl groups occur in closely placed pairs or clusters, increasing the probability of chelation, preference of the carboxyl groups for the alkaline earth cations supersedes that for the alkaline metal ions. As an example, ion-exchange resins with a very high density of carboxyl groups prefer Ca^{++} and Mg^{++} over the alkaline metal ions (30).

We may therefore expect that a high external concentration of Mg^{++} may or may not have an effect on the muscle cell resting potential, depending on whether the β - and γ -carboxyl groups on the muscle cell surface exist singly or in pairs or clusters.

Fortunately, this uncertainty in prediction has already been resolved by the earlier experimental finding that the pK value of the anionic sites of the frog muscle surface is 4.6, the same as that of "solitary" β - and γ -carboxyl groups⁽¹⁹⁾. If a significant fraction were in pairs or clusters, the pK would have been correspondingly higher^(31,32). These considerations led us to conclude that the surface β - and γ -carboxyl groups are indeed solitary and show little preference for Mg^{++} . As a result, the prediction of the association-induction hypothesis is diametrically opposed to that of the membrane pump theory of Hodgkin and Katz, i.e., the resting potential is expected to be insensitive to variations of external Mg^{++} concentration.

MATERIALS AND METHODS

All experiments were carried out on the isolated sartorius muscles of the North American leopard frog (Rana pipiens, pipiens, Schreber) from Vermont and occasionally from New Jersey. ²⁸Mg was obtained from Brookhaven Laboratory, New York (Lot 121779-7).

Resting Potential Measurements

Resting potentials of the sartorius muscles were measured using the method described by Ling and Gerard⁽³³⁾.

Composition of Mg^{++} -Ringer Solutions

To prepare a functionally "isotonic" high Mg^{++} -Ringer solution with varying Mg^{++} concentrations but a constant concentration of K^+ (25.0 mM) and of Na^+ (28.5 mM), two stock solutions were prepared: the high- Mg^{++} stock solution contained 24.6 mM $MgCl_2$, 36.6 mM $MgSO_4$, 2.5 mM KCl , 1.0 mM $CaCl_2$, 15.7 mM $NaHCO_3$, 217 mM NaH_2PO_4 , and 11.6 mM D-glucose in addition to 10% K^+ -free GIB medium, penicillin (0.1 mg/ml), and streptomycin (0.1 mg/ml)⁽³⁴⁾; the low- Mg^{++} stock

solution contained 147.5 mM sucrose, 23.2 mM D-glucose in addition to the same concentrations of KCl, CaCl_2 , NaHCO_3 , GIB medium, penicillin, and streptomycin as in the high- Mg^{++} solution. By mixing these stock solutions in different proportions, a series of Ringer's solutions containing different Mg^{++} concentrations ranging from 1.2 to 73.2 mM but constant concentrations of K^+ (2.5 mM) and of Na^+ (28.5 mM) were prepared (For further details, see ref. 16).

Measurements of Mg^{++} Permeability

We determined Mg^{++} permeability of frog muscles in two ways.

(1) From volume changes in isotonic MgCl_2 and MgSO_4 solutions. This is a very old method (51): cells are immersed in a Ringer's solution containing besides all its normal constituents at the normal concentrations, an excess of a high concentration of a solute, the permeability of which is being investigated. The cells promptly and rapidly shrink. If the cells are "impermeable" to the solute, the cell will, theoretically at least, remain shrunken indefinitely. On the other hand, if the cells are "permeable" to the solute, the cell will take up the solute and, in doing so, will regain its lost water. The rate at which the cell's weight is regained provides the basis for measuring the rate at which the solute enters the cell.

(2) From radioactive ^{28}Mg isotope efflux analysis. The basic method here is an extension of the technique extensively used in studying the efflux rates of Na^+ , sugars, etc. developed in this laboratory. Isolated and weighed sartorius muscles were equilibrated in Ringer solutions either at 25°C or 0°C in which the Mg^{++} has been labelled with ^{28}Mg . Connective tissues from areas adjacent to the sartorius muscle were isolated and similarly exposed to the labeled Ringer solutions and for the same lengths of time. At the conclusion of

incubation in the radioactively labeled Ringer solution, the muscles and connective tissues were placed between decks of partially wet filter paper, sealed hermetically in parafilm and centrifuged for 4 min. at 1000 g. Removed from the filter paper, the tissues were weighed in a torsion balance kept in a humidity chamber. Previous work has shown this centrifugation removes all the extracellular fluids and no significant amount of intracellular fluid of the sartorius muscle ⁽³⁵⁾.

The time course of labeled Mg^{++} efflux of the muscle and connective tissues were separately studied by washing the tissues in successive portions of Ringer solution having similar chemical composition but no radioactivity. After about $2\frac{1}{2}$ hours of washing, the muscles and connective tissues were blotted dry, reweighed, and its labeled Mg^{++} content analyzed. From the remaining labeled Mg^{++} found in the tissues of the radioactivities of the washing solution, the efflux curves were constructed. The efflux curves of the connective tissues from the same animals were then used to make corrections for the labeled Mg^{++} in the connective tissues of the sartorius muscles using the average volume of of the centrifuged connective tissue weights. The details of this technique were described elsewhere ⁽³⁶⁾. The corrected efflux curves plotted semilogarithmically are then resolved into its components by peeling off the slower exponential first, etc. From the slopes of the fraction their respective half-time of exchange and permeability constants were obtained.

RESULTS

Time Course of Swelling and Recovery of Frog Sartorius Muscles in Concentrated Solutions of $MgCl_2$ and KCl

Using the volume change method, we have made a study of the rate of entry of

MgCl₂ into isolated sartorius muscle cells. Figure 1 shows the typical pattern of initial rapid loss of weight followed by a slower regain. Curve B represents a muscle immersed in a Ringer's solution that contained an excess of 600 milliosmoles of MgCl₂ per liter; Curve A represents muscle immersed in a solution containing an excess of only 300 milliosmoles of MgCl₂ per liter. The half-time ($t_{\frac{1}{2}}$) of weight gain is about 70 min. for Curve B, while that for Curve A appears longer, owing in part at least to the merging of the shrinkage process with the initial re-gain in weight.

For comparison, Figure 2 represents a shrinkage-regain curve of a comparable experiment in which an excess of KCl instead of MgCl₂ was added. In this case, the half-time of regain ($t_{\frac{1}{2}}$) is approximately 180 minutes - more than twice as large as the $t_{\frac{1}{2}}$ of the MgCl₂.

We have elected to study the swelling-recovery method in order to leave little doubt that by any reliable method, high Mg⁺⁺ salt permeability is demonstrable. However, there are doubts that the rates for weight changes really reflect a permeability-limited process (see 37). Thus for more accurate and quantitative data we must turn to the radioactive tracer method to be described next.

Labeled Mg⁺⁺ Efflux Studies

Figures 3 and 4 show respectively the Mg⁺⁺ efflux from the centrifuged connective tissues and centrifuged sartorius muscles. The curves marked C were obtained after correction had been made for the connective tissue contributions and they are resolved into two fractions marked I and II. The slow fraction I has a half-time of exchange of 100 minutes or longer (A: 136; B: 148; C: 97 min.); its small intercepts (0.4 to 0.5 μ mole/g) suggest that this fraction had

only exchanged with the labeled Mg^{++} to a small extent. Of much greater interest here is the fraction II which represents the bulk of labeled Mg^{++} that has exchanged. Indeed the $t_{1/2}$ are from the three sets of data respectively 4.5 (A), 4 (B) and 4 (C) minutes. These and other data from three other sets of data are given in Table 1.

That the fast exchanging fraction with a $t_{1/2}$ of 4.7 min. is rate-limited by permeations through the cell surfaces agrees with the earlier demonstration that new equilibrium of Mg^{++} distribution is reached within 20 min ⁽¹⁶⁾. The present findings do not agree with the conclusion that Mg^{++} permeability is low in frog muscle ⁽¹⁵⁾. On the contrary, it is very rapid as Tasaki et al ⁽¹⁴⁾ demonstrated for Ca^{++} permeability in squid axons.

The outward rate constant of Mg^{++} efflux, $k_{outw} = \frac{\ln 2}{t_{1/2}}$. The outward permeability constant, κ_{outw} is equal to k_{outw} divided by the surface-volume ratio (A/V) which equals $550 \text{ cm}^2/\text{g}$ for frog muscle (4, p. 208). From the data of $t_{1/2}$ given in Table 1, one obtains an average κ_{outw} equal to $\frac{0.693}{4.7 \times 550} = 2.68 \times 10^{-4} \text{ cm sec}^{-1}$. At equilibrium the inward flux (M_i) and outward flux (M_o), defined as the number of moles per cm^2 per second must be equal.

$$\text{Since } M_o = M_i, \quad (3)$$

$$M_o = \kappa_{outw} [Mg^{++}]_{int}, \quad (4)$$

$$M_i = \kappa_{inw} [Mg^{++}]_{ex}, \quad (5)$$

where $[Mg^{++}]_{int}$ is the intracellular free Mg^{++} concentration, or interstitial Mg^{++} concentration and $[Mg^{++}]_{ex}$ is the extracellular Mg^{++} concentration,

$$\kappa_{inw} = \frac{[Mg^{++}]_{int}}{[Mg^{++}]_{ex}} \kappa_{outw}. \quad (6)$$

From an earlier publication, we know that at equilibrium (25°C), the Mg equilibrium distribution coefficient, is

$$q_{Mg} = \frac{[Mg^{++}]_{int}}{[Mg^{++}]_{ex}} = 0.206 . \quad (7)$$

Thus $\kappa_{inw} = 0.206 \times 2.68 \times 10^{-4} \text{ cm/sec} = 0.552 \times 10^{-4} \text{ cm/sec}$. Next we shall calculate the permeability constant for Mg^{++} , P_{Mg} , according to the model of Hodgkin-Katz-Goldman (38, p. 60).

$$P_{Mg} = \frac{\kappa_{inw}}{\phi} , \quad (8)$$

where the factor

$$\phi = \frac{Z\psi F/RT}{1 - \frac{1}{\exp(Z\psi F/RT)}} , \quad (9)$$

where Z , the valency is 2; ψ , the resting potential, is equal to 86 mV (see below) and positive. R and T are the gas constant and absolute temperature respectively. At 25°C, RT is equal to $8.614 \times 10^{-5} \times 298 = 2.567 \times 10^{-2}$ volt-Faraday or roughly 26 mV-Faraday.

$$\phi = \frac{2 \times \frac{86}{26}}{1 - \left(\frac{1}{\exp(\frac{86}{26})} \right)} = 6.62 . \quad (10)$$

From Equation 8, we find

$$P_{Mg} = \frac{0.552 \times 10^{-4}}{6.62} = 83.4 \times 10^{-7} \text{ cm sec}^{-1} \quad (11)$$

The Mg^{++} permeability constant P_{Mg} calculated according to Hodgkin-Katz-Goldman is then compared with similarly calculated P_K in Table 2. Note that P_{Mg} is more than 5 times higher than P_K .

Effect of Varying External Mg^{++} Concentration upon the Resting Potential of Frog Sartorius Muscle

Two sartorius muscles were introduced into flasks containing 25 ml of each of the solutions with different Mg^{++} concentrations and were incubated at $4^{\circ}C$, and the flasks were shaken gently for 18 hours. At the end of this period, the flasks were warmed to room temperature before the resting potentials of at least 8 single muscle fibers (four from each muscle) were measured while the muscles were immersed in an aliquot of their respective incubation solutions. The result is shown in Figure 5. No significant depolarization of the resting potential was observed in response to increasing external Mg^{++} concentration from a concentration of 1.2 mM to 73.2 mM.

Effect of Varying Duration of Exposure to High External Mg^{++} Concentration at $25^{\circ}C$ upon Resting Potential

The data of Figure 5 were obtained after prolonged exposure of frog muscles to the experimental solutions at $4^{\circ}C$ followed by warming to $25^{\circ}C$. Figure 6 shows the observed indifference of the resting potential to the high external Mg^{++} concentration was the same when the temperature was maintained at $25^{\circ}C$ throughout the incubation period of over 15 hours.

The promptness with which the sartorius muscles responded to high external

K^+ concentrations has long been known (3 ; 4, p. 277). In this case, a new reduced level of potential was reached in the high K^+ solution within a matter of 1 or 2 minutes and remained there for at least 10 hours without further change. The difference between the effectiveness of high external K^+ in reaching the potential and the total ineffectiveness of similar high concentrations of external Mg^{++} in reducing the potential could not be more striking.

The demonstration that Mg^{++} permeability constant calculated according to Hodgkin and Katz is more than 5 times larger than K^+ permeability constant and yet at high concentrations produces no effect on the resting potential offers strong evidence against the membrane-pump model of the cellular potential. The same findings also offer evidence in support of the association-induction hypothesis.

The observed sensitivity to external alkali-metal ions and insensitivity to external Mg^{++} correlates the behavior of the frog muscle cell surface to a model that was introduced years ago, i.e., oxidized collodion-coated glass electrodes⁽²⁹⁾. This model exhibits similar electrical behavior (sensitivity to K^+ , lesser sensitivity to Na^+ and insensitivity to Mg^{++}) as a result of the introduction of carboxyl groups onto its surface. These findings support the theory that the muscle cell surface is endowed with primarily solitary β - and γ -carboxyl groups and that these fixed anionic groups are responsible for both the polarity and the magnitude of the resting potential of frog muscle cells.

This set of experimental findings and conclusions should be viewed with a number of other developments which are almost unanimously in harmony with the conclusion from the present study. And it is perhaps timely to review them briefly.

There are seven variables in the Hodgkin-Katz equation: T , $[K^+]_{ex}$, $[Na^+]_{ex}$, $[Cl^-]_{ex}$, $[K^+]_{in}$, $[Na^+]_{in}$, and $[Cl^-]_{ex}$. The predicted relationship between ψ and the first three variables has been repeatedly confirmed. However, this cannot be said for the predicted relationship between ψ and the last four variables because a lasting change of the potential with variations in $[Cl^-]_{ex}$ was not observed ⁽³⁹⁾; and although a confirmation of the predicted relationship between $[K^+]_{in}$ perfused squid axon and ψ was reported ⁽⁴⁰⁾ but the slope of ψ vs. $\ln [K^+]_{in}$ plot has a much lower value than required. Furthermore this confirmation was directly contradicted by a subsequent report from another laboratory in which no change of potential was observed in perfused squid axons when a perfusant solution of K_2SO_4 was changed to that of Na_2SO_4 ⁽⁴¹⁾.

In addition, no less than six other sets of independent experimental observations have been reported; all of which are in agreement with each other but not with the theoretical predictions of a relation between $[K]_{in}$ and ψ ⁽⁴²⁻⁴⁸⁾. Two of these papers also report failure to detect the predicted relationship between $[Na^+]_{in}$ and ψ during nerve or muscle activity ^(42,44,45).

If an equation is derived rationally it must have an internal coherence. When experimental studies failed to confirm the predicted relationship between four of the seven variables and ψ , the basic theory is probably incorrect. However, the association-induction hypothesis offers equations for ψ (Equations 2 and 3) that contain no more than the three variables whose relationships to ψ have already been verified.

It is to be noted that the association-induction hypothesis agrees well not only with the data that support the membrane theory, but also with the data which do not support the membrane theory. Thus, the association-induction

hypothesis does not predict a dependence of the potential upon external anions (e.g., chloride) and, in fact, none was found. The association-induction hypothesis also does not predict a mandatory dependence on intracellular ions, and, again, none was consistently found (for further discussion, see 6,7).

Two other new findings bear on the critical subject of a choice between the two alternate models; in both, the young German scientist, Ludwig Edelmann, made a major contribution (45).

If one keeps $[Cl^-]_{ex}$, $[Cl^-]_{in}$, $[K^+]_{in}$, and $[Na^+]_{in}$ essentially constant, say, in a short-term experiment, Equation 1 can be written as

$$\psi = \text{constant} - \frac{RT}{F} \ln \left\{ P_K [K^+]_{ex} + P_{Na} [Na^+]_{ex} \right\} . \quad (12)$$

Equation 12 is formally identical to Equation 2 except that the coefficients of the external concentrations have different meanings. The P's are permeability constants for K^+ and Na^+ , and the \tilde{K} 's are adsorption constants of these ions on surface anionic sites. Edelmann, having noted this fundamental difference, designed an experiment to test the two alternate theories. He concluded that the cellular electrical potential has no relation to permeability but represents adsorption constants, as described by Equation 2 (45).

An even more recent finding is that the bulk of intracellular K^+ is not freely distributed in the cell water (21-26). In skeletal muscles, intracellular K^+ is localized primarily in the A-bands and the Z-line, in agreement with the association-induction hypothesis, which has long contended that intracellular K^+ is selectively adsorbed on the β - and γ -carboxyl groups of cellular proteins. This follows from the fact that most muscle β - and γ -carboxyl groups are carried

by the myosin that makes up the A-band ⁽²²⁾ and from the fact that β - and γ -carboxyl groups bind uranium in EM plates of fixed cell sections ⁽⁴⁹⁾, and that uranium is concentrated on the A-band, Z-line, etc. ⁽⁵⁰⁾.

The demonstration of the adsorbed state of intracellular K^+ , on one hand, makes the Hodgkin-Katz equation untenable, since this equation was based on the assumption that virtually all intracellular K^+ exists in the free state. On the other hand, this demonstration affirms the association-induction hypothesis in a most basic manner, since in this hypothesis the electrical potential should have no direct causal relationship to the bulk-phase intracellular K^+ , its concentration, or its state of adsorption.

Therefore, the finding presented here of the independence of ψ with relation to external Mg^{++} is not an isolated observation but is one of many findings contradicting the membrane theory and supporting the association-induction hypothesis.

ACKNOWLEDGMENTS

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LEGENDS

- FIGURE 1. The time course of weight change of a frog sartorius muscle after immersion in a Ringer-GIB medium containing, in addition to the normal constituents at their respective normal concentrations, 100 mM MgCl_2 (top curve) and 200 mM MgCl_2 (bottom curve). The muscle was weighed at intervals after blotting on moist filter paper.
- FIGURE 2. The time course of weight change of a frog sartorius muscle after immersion in a Ringer-GIB medium containing, in addition to the normal constituents at their respective normal concentrations, 150 mM KCl (top curve) and 300 mM KCl (bottom curve). The muscle was weighed at intervals after blotting on moist wetted filter paper.
- FIGURE 3. Four curves of labeled Mg^{++} from isolated frog "connective tissues." Connective tissues from 4 different frogs were incubated and washed in the same manner as frog sartorius muscles and these curves are used to make corrections for contribution of similar connective tissues in the sartorius muscle efflux curves (see legend of Fig. 4). Connective tissues represent loose connective tissues and small nerves, blood vessels, and other non-muscle cell tissues.
- FIGURE 4. The efflux curves of labeled Mg^{++} from 3 frog sartorius muscles. Muscles were incubated in a Ringer solution containing 78 mM labeled Mg^{++} for 21 min at 25°C and centrifuged to remove extracellular space. Washout was carried out in a similar Ringer solution without ^{28}Mg and also at 25°C . Curves marked C were obtained after correcting for connective tissue contribution but without further calculation to change the unit of concentration to the base of pure cell

weights. The corrected curves were resolved into two fractions (I, and II). The $t_{1/2}$'s of the fast fraction (II) correspond to the time it took for radioactively labeled Mg^{++} belonging to that fraction to fall to $\frac{1}{2}$ of its initial value.

FIGURE 5. The effect of external Mg^{++} concentration upon the resting potential of isolated frog sartorius muscles. The muscles were exposed to Ringer's solution with varying concentrations for 18 hours at $4^{\circ}C$ followed by warming to $25^{\circ}C$ before measurements of resting potential were made.

FIGURE 6. The graph shows that the resting potential is independent of the prolonged exposure to a high Mg^{++} Ringer solution. The entire experiment was carried out at $25^{\circ}C$.

TABLE 1. The half time of exchange of labeled Mg^{++} from isolated frog sartorius muscle at $25^{\circ}C$. Isolated sartorius muscles were incubated for different lengths of time at $25^{\circ}C$ as indicated. The $t_{1/2}$ of exchange was estimated from the fast fraction, i.e., Fraction II as in Fig. 4.

TABLE 2. A comparison of the Hodgkin and Katz permeability constant (P_i) of K^+ and Mg^{++} for frog muscles.

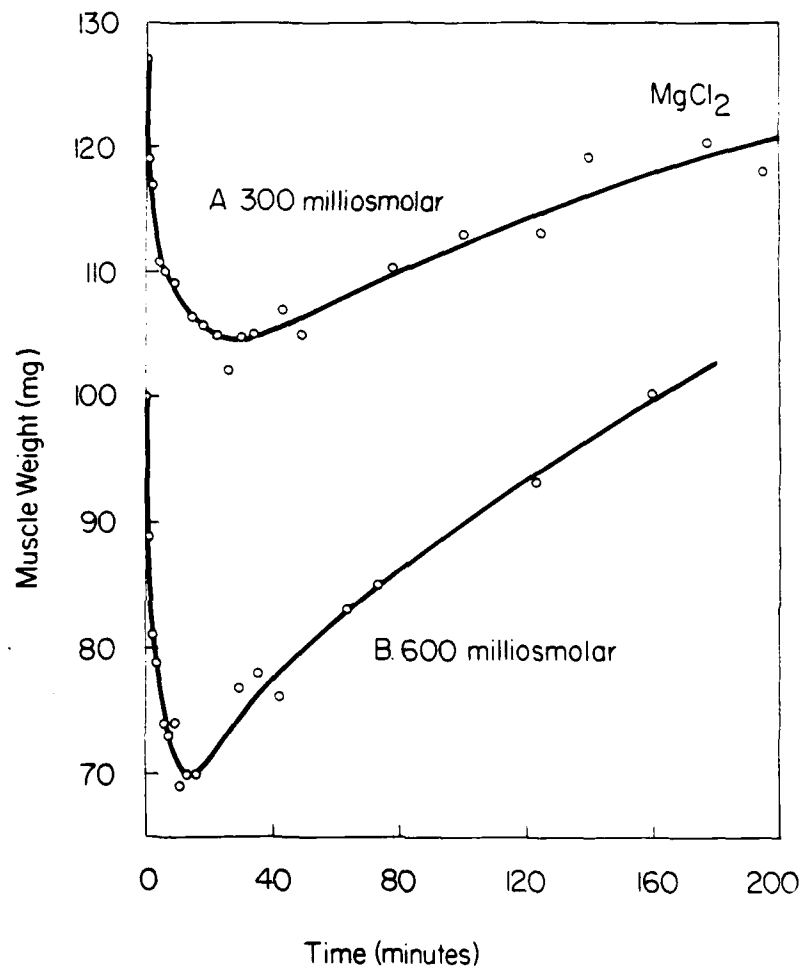


FIGURE 1

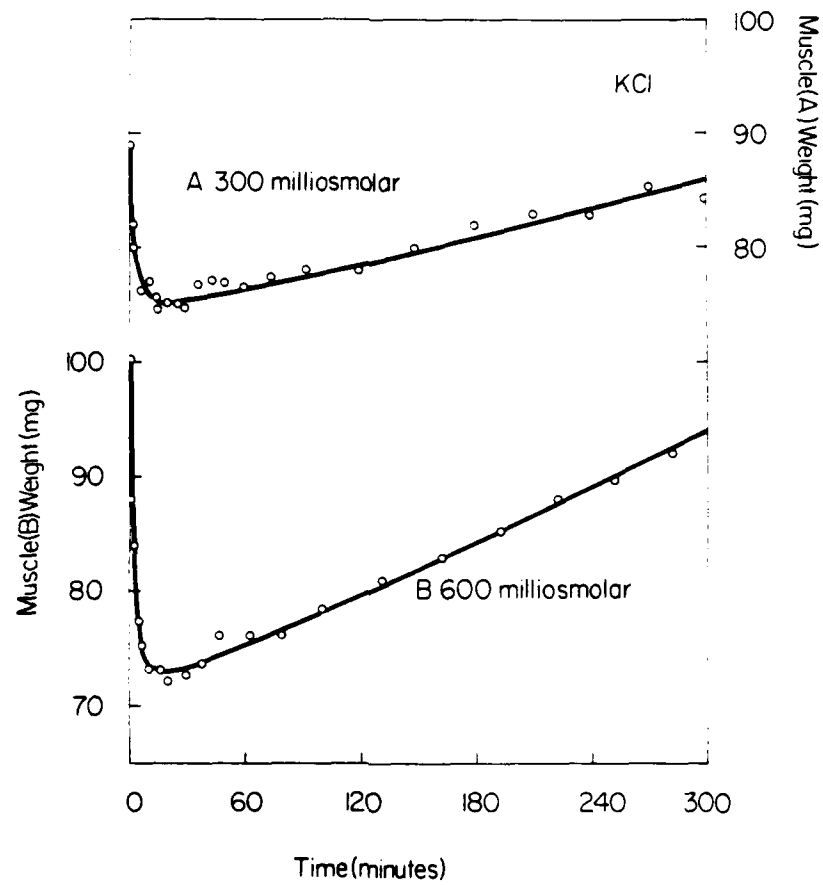


FIGURE 2

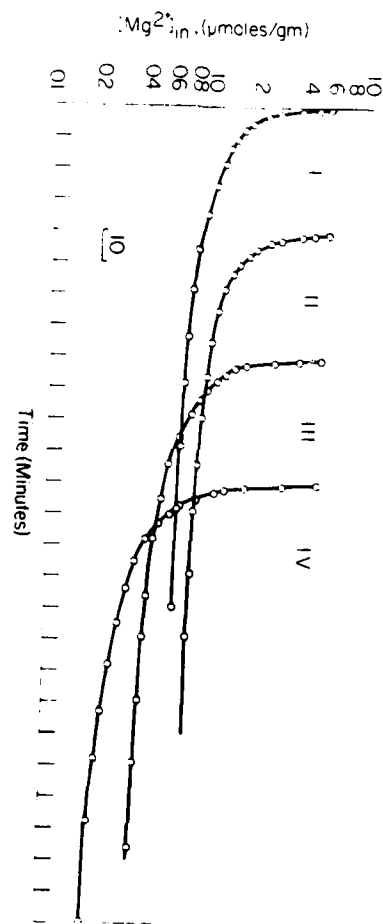


FIGURE 3

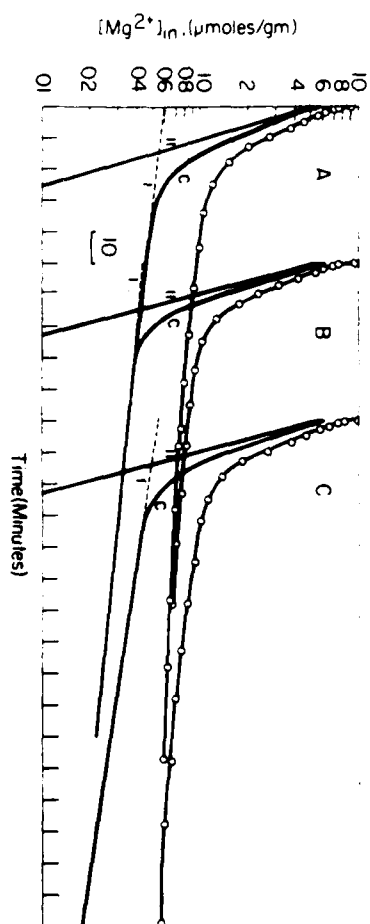


FIGURE 4

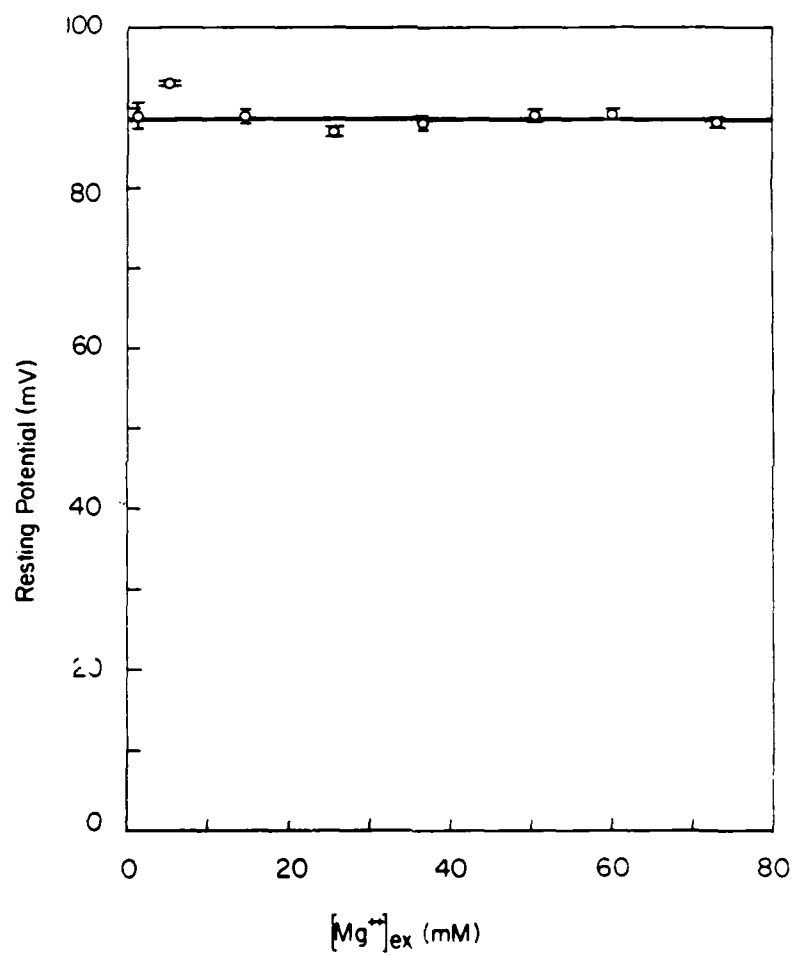


FIGURE 5

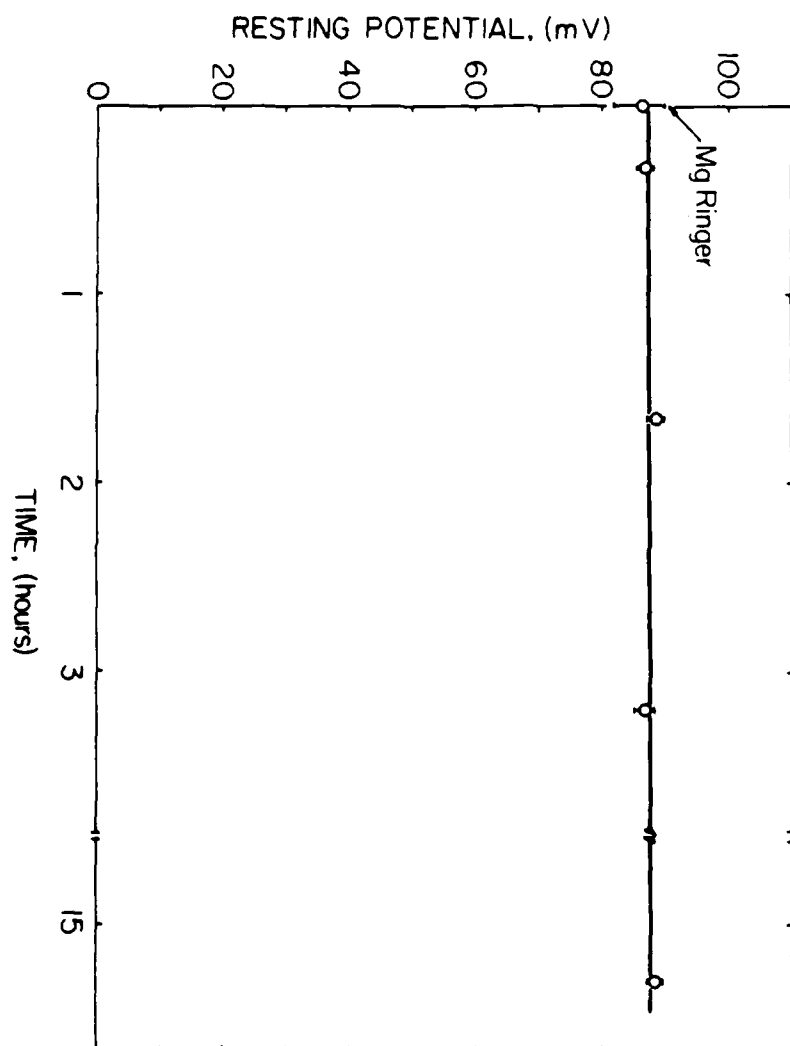


FIGURE 6

<u>Date</u>	<u>Experiment No.</u>	<u>Incubation Duration (min)</u>	<u>t_{1/2} of Fast Fraction (min)</u>
12-18-79	A	21	4.5
	B	21	4.0
	C	21	4.0
	E	10	5.0
	F	10	4.8
	H	10	7.0
	M	11	5.0
	N	11	4.8
	O	11	4.8
3-18-80	I	62	3.5
	J	62	3.6
	K	62	5.0
mean \pm S.E.			<u>4.7 \pm 0.25</u>

TABLE 1

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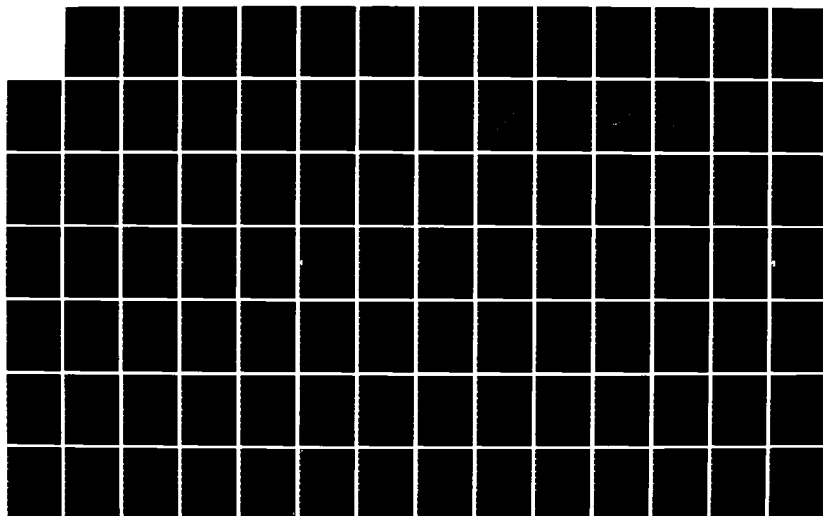
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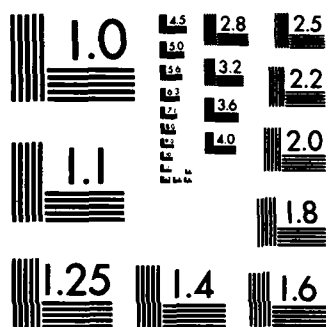
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MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

<u>Ion</u>	<u>Author</u>	k_2 <u>(hr⁻¹)</u>	<u>Average</u>	P_i <u>(cm/sec)</u>	<u>Relative Permeability</u>
K ⁺	Mullens, 1959	1.92	2.97	9.69 X 10 ⁻⁷	1
	Ling, 1962 (25°C)	3.06		15.4 X 10 ⁻⁷	
	Ling and Ochsenfeld, 1965 (24°C)	3.10		15.6 X 10 ⁻⁷	
				<u>21.1 X 10⁻⁷</u>	
	Katz, 1966	3.80		15.4 X 10 ⁻⁷	
Mg ⁺⁺	(present paper)			83.4 X 10 ⁻⁷	5.4

TABLE 2

COLD INJURY-INDUCED BRAIN AND OTHER TISSUE SWELLING AND ITS MOLECULAR MECHANISM

by

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Cell swelling and shrinkage were among the first physiological manifestations of the living cell that lent themselves to quantitative measurements. Studies of these cell volume changes had led to the founding of the first major theory in cell physiology - the membrane theory. While the early laboratory manipulations that produced these volume changes were usually achieved by altering the osmotic activity of the surrounding fluids, it also became known that within an environment with an osmotic activity equal to that of the cell interior, cells can undergo extensive swelling as they do in an isotonic KCl solution (von Kőrös, 1914) or in response to exposure to 0° C temperature while in its natural isotonic environment rich in NaCl (Stern, et al, 1949; Opie, 1949). Indeed that injury can lead to tissue swelling is also well known to physicians and lay people. Injury-induced swelling of brain is especially dangerous and sometimes fatal due to the confinement of the brain in a rigid box - the cranium.

Early theory of cell swelling and shrinkage was based on the assumption that the cell membranes were impermeable to solutes that can cause and maintain the shrunken state of the cells. A prime example of the impermeant solute thus defined was the Na^+ ion. This theory was disproven when it was shown that Na^+ is in fact permeant to the cell membrane (Cohn and Cohn, 1939; Heppel, 1940). The Na^+ pump was then postulated to maintain a low level of Na^+ in the cell and hence a constant cell volume (Lilly, 1923; Dean, 1941). Swelling due to injury or metabolic interference has been attributed to an interference with the functioning of the metabolic pumps, either continually driving out water (Opie, 1954; Robinson, 1952) or Na^+ (Tosteson, 1964). However, these theories became questionable when it was shown that the Na^+ pump alone would consume energy 15 to 30 times that available and that the swelling

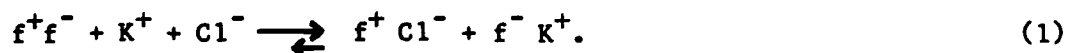
of cells in hypotonic Ringer solution or in isotonic KCl remained unchanged after the intactness of the cell membrane was destroyed by cutting the cell into small segments (Ling and Walton, 1976). Under this condition, it was clearly established that there was no regeneration of a new membrane at the sites of amputation (Ling, 1973).

The basic concepts that swelling behaviors of living cells may reflect the whole protoplasm rather than primarily the cell membrane can be traced back to Martin Fischer (1909). In more recent time, the association-induction hypothesis has presented a unified conceptual framework to explain different types of swelling phenomena (Ling, 1962, 1969). The basic assumption, now supported by considerable experimental evidence (Ling, 1984), is that the osmotic activity of living cells (which is a measure of the reduction of the activity of cell water) is only to a minor extent provided by free intracellular ions and other solutes, notable K^+ . This is so because virtually all of the cell K^+ , the major cation found in the cell, (as well as most of the intracellular anions) exists in an adsorbed state (Ling, 1977a, Edelmann, 1977, 1981; Trombitas and Tigyi-Sebes, 1979). In this theory the reduction of cell water activity, measured as osmotic activity, is due primarily to certain cellular proteins, called matrix proteins. These matrix proteins exist in an extended conformation with their polypeptide NH and CO groups directly exposed to, and polarizing the bulk of cell water (Ling, 1965, 1972, 1979). Water so polarized has reduced activity and exhibits osmotic activity far beyond its molar concentration predicted on the basis of Boyle-van't Hoff Law (Ling, 1980, 1983). Experimental confirmation that water so polarized does indeed have high osmotic activity has just been published (Ling, 1983).

To put it differently, the behaviors of water in living cells reflects

its existence in the state of polarized multilayers. In support, Ling and Negendank (1970) have demonstrated that 95% of the water in isolated frog muscles follows Bradley's multilayer-adsorption isotherm. Simple osmotic swelling in hypotonic solution is due to the higher water activity in a hypotonic solution

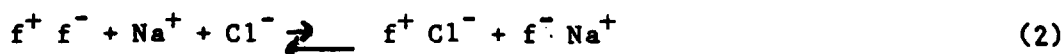
and the natural tendency for water to seek the phase of lower water activity. Shrinkage in hypertonic solution on the other hand, is just the opposite. To explain swelling induced by an isotonic KCl solution, a more complex interpretation is involved: As a rule normal cells have a tendency to swell but this swelling tendency is held back due to the presence of salt linkages which are formed between negatively charged side chain functional groups (e.g., β - and γ -carboxyl groups) and positively charged side chain functional groups (e.g., ϵ -amino and guanidyl groups) of neighboring protein chains in the cells. Thus under normal conditions salt-linkages between adjacent intracellular protein molecules prevent the full amount of water to be taken into the cell and adsorbed. However, KCl can split these salt linkages, as follows (Ling and Peterson, 1977):



It was shown that this dissociation is autocoperative, i.e., all-or-none. That is, the salt linkages (f^+f^-) formed between fixed cations (f^+) and fixed anions (f^-) will resist the dissociative action of KCl until KCl reached a certain critical concentration. At this concentration, the salt linkages would all split up and the cell undergoes a step-wise swelling. As a rule, as one increases the KCl concentration gradually in the external medium, the cell undergoes several step-wise swelling, interspersed with shrinkage due to the increase of total osmotic activity in the external medium with increase of total KCl concentration. As mentioned above, after the intact muscle

cells were cut into small segments 2 or 4 mm long with no membranes at the ends of these segments, 100 mM KCl in the external medium will continue to induce swelling while 100 mM NaCl will not (Ling and Walton, 1976).

According to the association-induction (AI) hypothesis, this selective sensitivity to KCl but not to NaCl reflects a high preference of the fixed anions (f^-) or more specifically, the β - and γ -carboxyl groups of the proteins to adsorb K^+ over Na^+ . In other words the reaction represented by Equation 1 goes strongly to the right, while reaction represented by Equation 2 below



goes only weakly to the right. In the AI hypothesis this cationic selectivity reflects a specific electronic density at the β - and γ -carboxyl groups, represented by the c -value (Ling, 1960, 1962). Figure 1 reproduces the theoretical curves showing that when the c -value is, say, around -3.5 \AA , K^+ is greatly preferred over Na^+ in the selective adsorption onto the β - and γ -carboxyl groups.

The AI hypothesis suggested that since the fixed cations like α -amino groups, ϵ -amino groups and guanidyl groups are all modified NH_4^+ , the preference of some fixed β - and γ -carboxyl groups to form salt linkages may have the same cause why K^+ is preferred over Na^+ by other β - and γ -carboxyl groups not engaged in maintaining the cell volume but engaged in selective accumulation of K^+ in the cells. As Fig. 1 shows, NH_4^+ and K^+ follow very similar courses of changes with c -value changes. At the c -value of -3.5 \AA , NH_4^+ has a much more favorable adsorption energy than Na^+ just as K^+ does.

Another major postulate of the AI hypothesis is that the c-value of the β - and γ -carboxyl groups functions together in unison due to autocoooperative interaction among these sites, and that normal c-value of those sites as described above are under the control of cardinal adsorbents including ATP (for evidence, see Ling, 1977b). Evidence has also been presented showing that the function of ATP is to lower the c-value (Ling, 1981a, p. 86). Thus, if the concentration of ATP is reduced as a result of metabolic interference by cold or injury, the c-value may go to a higher value, say -2.4 \AA (Fig. 1). At this higher c-value, the theoretical curves show that K^+ and Na^+ are equally preferred at the f^- sites. Indeed there are evidence that interference with metabolism and the consequent ATP depletion does bring about a depolarization of cell water and an increase of free Na^+ as well as a transient increase of adsorbed Na^+ (Ling and Ochsenfeld, 1973; Ling et al, 1981).

As mentioned above, under normal conditions all cell ATP is adsorbed on cardinal sites on cellular proteins. This is a theoretical postulate by the AI hypothesis but it is made inevitable by the enormous binding constants of ATP on the cell protein, myosin ($3.25 \times 10^{11} M^{-1}$, Goody et al, 1977; Cardon and Boyer, 1978). The c-values of the f^- engaged in the salt linkages are low so that only KCl can cause the dissociation of those salt linkages and cell swelling, NaCl cannot. However, when ATP is depleted, the c-value of f^- rises. Now the normally ineffective 100 mM NaCl present in the Ringer solution or plasma, suddenly becomes as powerful as 100 mM KCl in causing f^-f^+ dissociation and cell swelling follows. This then is the theory of injury-induced swelling according to the AI hypothesis. The AI hypothesis has offered, for the first time, a theoretical connection between swelling of

normal cells in isotonic KCl solution and of cells injured by colds or poisons in normal plasma or Ringer solution. The present communication represents experiments designed to test this theory.

MATERIALS AND METHODS

All experiments were performed on isolated organs of Swiss (ICR) mice. Isolated brain, kidney are usually cut sagittally into halves, weighed on a torsion balance before being introduced into 5 ml of the experimental solution in a closed vial (to be described). These vials were kept at 4° C in a cold room without shaking. The tissues were taken out 24 hours latter, blotted lightly and weighed ahen and on every day following for as many as six days. Low temperature combined with the antibiotics added kept bacteria from growing.

Table 1 gives the basic formula for the solutions used. Two major stock solutions (I and II) were each prepared by mixing three solutions (IA, IB, IE, etc.) which were kept apart to prevent precipitation and deterioration of the organic components in the GIB medium. The GIB medium was obtained in sterile dry powder form from Grand Island Biologicals (now GIBCO, Wilmington, DE). It is a chemically defined medium (for composition see Ling and Bohr, 1969).

Determination of ATP was by a firefly method essentially that described by Kahlhen and Koch (1967). The only significant modification was that the tissue extract was made with a 5% TCA rather than boiling water. The light emitted by the luciferin-luciferase system in the presence of ATP was measured in a β -scintillation counter.

RESULTS

When isolated mouse kidney tissues were incubated at 4°C in a normal mammalian Ringer solution containing about 120 mM Na^{+} it swelled up rapidly in the first day. Intact kidneys increased their weights for another day before a gradual decline (Fig. 2). On the other hand, if kidney halves

were measured, the peak height of weight change was reached at one day and the decline was earlier. Since as a rule half organs were used, we chose one day equilibrium time as a rule. Half mouse brains, however, were an exception. It took two days to reach its maximum weight and then remained at these peak values for two more days. For this reason the brain weight was obtained at two to four days.

Figure 3 shows the weights of three mouse tissues after one day incubation at 4°C in various solutions containing different proportion of NaCl and sucrose. The ordinate represents the one day weight represented as the ratio of the final weights, W_{fin} divided by the initial weight (W_{init}). In spite of the fact that all solutions used were isotonic, marked difference in $W_{\text{fin}}/W_{\text{init}}$ occurred. In all cases the highest ratios were those organs in the solution containing the most NaCl and the least sucrose. However the degree of swelling in the presence of the high NaCl concentration varied considerably among the three tissues studied here. They fall in the rank: kidney > liver > spleen.

Also clearly notable is the considerable shrinkage for all three organs after exposure to the Na-free sucrose Ringer solution represented by the left-hand most point along the abscissa. However, this shrinkage in Na-free sucrose Ringer solution is not universal. Other tissues like the brain did not undergo shrinkage in any solution studied (see below).

Figure 4 compares the shrinkage-swelling curves of mouse kidney in various solutions in which the sucrose components (Stock Solution II) were similar but the NaCl concentrations were either the same as in the experiments described in Fig. 3 or it was replaced by an isoosmotic equivalent of LiCl, Li_2SO_4 , or MgSO_4 . LiCl and MgSO_4 replaced NaCl mole for mole. On the other hand, each mole of NaCl was replaced by 2/3 M of Li_2SO_4 .

Note that mole for mole, LiCl seemed even more effective than NaCl. Yet Li_2SO_4 was much less effective, suggesting that the swelling depends not only on the specific nature of the cation but also that of the anion as well. The virtually complete ineffectiveness of MgSO_4 further confirms this view. The kidney consistently shrank in the sucrose-Ringer.

Figure 5 shows the swelling of mouse brains in NaCl and Na_2SO_4 Ringers and in LiCl and in Li_2SO_4 respectively. The greater swelling in the chloride salt than the sulfate salt is in full agreement with the conclusion for the kidney study. In contrast to kidney, liver and spleen, however, brain tissues did not undergo shrinkage in the sucrose Ringer solution. Figure 6 shows that LiCl caused more swelling than isoosmotic concentrations of Li_2SO_4 .

In Figure 7 we have plotted the water contents of half brains against the ATP concentrations measured in the tissues after they have been incubated at 4° for various lengths of time in hours, as indicated by the number in brackets beside each experimental point. The incubation solution used contained the full amount of NaCl of a normal mammalian Ringer (118 mM). The data shows that the swelling of the brain in the presence of a high concentration of external NaCl follows closely the decline of the ATP contents of the tissue.

DISCUSSION

According to the AI hypothesis, water in resting, normal living cells is not free water in which free K^+ salts are dissolved. Rather, water exists in the cells in the state of polarized multilayers. Most of the intracellular ions, of which K^+ is predominant, is adsorbed singly on anionic protein sites (Ling, 1981b). The maintenance of the normal amount of cell water reflects the balance of two opposing forces: expansive force originates from the tendency of the cells to acquire additional layers of water and restrictive bonds between neighboring protein chains, largely in the form of salt-linkages (Ling and Peterson, 1977). When the cardinal adsorbent ATP is present at normal level and all the cardinal sites for ATP, Ca^{++} , etc. fully occupied, KCl (and RbCl) at about 100 mM concentration causes extensive swelling because free-energy wise it is more favorable for the reaction described in Equation 1 to proceed to the right. In contrast, isoosmotic concentration of sucrose is totally ineffective. K_2SO_4 or NaCl are also ineffective or less effective. The sulfate ion is known to be less strongly adsorbed than Cl^- on the ϵ -amino group and guanidyl group (see Ling, 1962, p. 172). Na^+ and Li^+ are less preferred by the fixed β - and γ -carboxyl groups when these groups are held at a relatively low c-value at which K^+ , NH_4^+ , and the variant of NH_4^+ , the α -amino, ϵ -amino, and guanidyl groups are preferred. Most of these theoretical expectations have been verified elsewhere experimentally (Ling and Peterson, 1977).

According to the AI hypothesis, any kind of metabolic interference or injury to the living cell whether brought on by chilling, mechanical damage or metabolic poison, usually leads to a diminished ability of the cell to regenerate ATP and thus to keep its cardinal sites occupied by this critically important cardinal adsorbent. Clearly incubating isolated mouse tissue at a

low temperature without its normal efficient supply of oxygen and other essential exchange of nutrients and water lead to gradual ATP depletion as Fig. 6 shows. We would also like to suggest that the observed swelling of slices of liver, kidney, and brain mentioned earlier by exposure to 0°C temperature, metabolic inhibitors like cyanide and 2,4-dinitrophenol, and various mercurials produced NaCl dependent swelling by a similar mechanism.

Let us next discuss the special requirement of NaCl, the major component of the blood plasma for the injury-induced swelling.

Figures 3 to 6 show quite consistently that tissues exposed to low temperature in Ringer solution whose NaCl had been replaced by an isoosmotic concentration of sucrose, exhibited little swelling. Indeed, often there was shrinkage. This special requirement for Na^{+} in injury-induced swelling was recognized by Saladino, Bentley, and Trump (1969). One asks, "Is this substitution of two moles of sucrose for one mole of NaCl truly accurate enough in terms of osmotic activity?" The answer is yes. Thus Negus (see Fraser, 1927) gave the partial vapor pressure of 0.15 M NaCl as 0.995. The partial vapor pressure of 0.30 M sucrose is also 0.995 (Bear, see Fraser, 1927). Clearly the widely different effects of sucrose Ringer solution and NaCl Ringer solution on swelling is not a simple matter of osmotic activities of the two solutions, which are in fact identical. Nor can this special requirement for NaCl be one due to the net electric charges of the particles Na^{+} and Cl^{-} . Thus isoosmotic MgSO_4 bearing net charges is nearly as ineffective as isoosmotic sucrose bearing no net charges in causing injury-initiated swelling of mouse kidneys.

The failure of sucrose as well as MgSO_4 to produce swelling is a clear affirmation of a high degree of specific (Na^{+}) preference in the reaction

described by Equation 2 just as there is a high degree of specific K^+ preference in the reaction described by Equation 1, which is applicable to normal rather than injured tissues.

That Mg^{++} cannot replace Na^+ in causing swelling is first and foremost a question of relative adsorption energy. As pointed out earlier the Mg^{++} and other divalent cations are ineffective in the depolarizing of the electrical potential of living muscle cells and model systems (Ling, Walton, and Ling, 1979). The interpretation was that divalent ions are only very weakly adsorbed onto isolated single β - and γ -carboxyl groups which adsorb monovalent cations. We believe that in all probability this is the case here. According to this theory, if the β - and γ -carboxyl groups occur in pairs or clusters, a different observation would have been made. Namely Mg^{++} salts would be as effective as or more effective than Na^+ salts in causing swelling. But clearly, this is not the case here.

The ineffectiveness of $MgSO_4$ in causing swelling is partly also due to the ineffectiveness of SO_4^{2-} . That SO_4^{2-} is as a rule very weakly adsorbed on amino types of cationic groups has been emphasized often in the presentation of the AI hypothesis and is fully borne out by the comparative study of the relative binding energies on weak amino type of ion exchange resins and on isolated proteins (Ling, 1962, p. 172).

Another strong support for the theoretical interpretation offered here for injury-induced swelling is the effectiveness of $LiCl$ in promoting injury-induced tissue swelling. An examination of the theoretical plots of c-value vs. relative adsorption energy on fixed anionic groups in Fig. 1 shows that the observation is theoretically predicted: at such a c-value when Na^+ is effective in causing swelling, Li^+ should also be effective.

We want to ask next why do injured tissues actually undergo shrinkage in sucrose Ringer solution. This is not the first time that isoosmotic sucrose-Ringer solution was found to induce cell shrinkage. Ling, Walton, and Ling (1979) noted the same phenomenon in normal, uninjured frog muscles when NaCl in a normal Ringer solution was replaced by an isoosmotic concentration of sucrose. In answer, the following explanation is offered: In normal cells, a small number of salt linkages are kept dissociated by the NaCl as illustrated in Equation 2. It is possible that the anionic components of these salt linkages are different from the majority of their counterparts and have a higher c-value at which Na^+ is preferred than at most of the other sites so that in the presence of 100 mM or so of NaCl in the normal environment the NaCl is able to sustain the dissociated state of these particular salt linkages and allow a corresponding number of multilayers of water to exist in the cells. When this NaCl is replaced by sucrose, the reaction represented by Equation 2 goes to the left and the cell then loses water as observed.

Finally we would like to point out that in the combined ischemia- and cold-induced injury seen here, the initial swelling may well be reversible (see Stern et al, 1949; Robinson, 1950; Whittam and Davies, 1953). But as time progresses, the cell certainly became irreversibly damaged. Thus the molecular mechanism as represented by Equation 2 is more fully applicable to the initial state of swelling. During this stage, the polarized multilayered state of water is still maintained. Augmented by the liberated adsorbed ions, the high total osmotic pressure provides the basic force for swelling when the restraining force of salt linkages are simultaneously broken down by the NaCl present. However, this process would continue only for a certain length of time. As ATP begins to reach very low concentra-

tion, cell water depolarizes and the initial excessive osmotic activity of the injured cells due to the combined contribution to the osmotic activity of polarized water and liberated K^+ declines. Although still apparently swollen, this state is only the remnant of the swollen state: extended, overblown "shells" filled with free water and free salt ions. In support of this view, Ling and Walton (1976) had shown that centrifugation for 4 minutes at 1000 g. of living frog tissues whether intact or cut into segments remove only water in the extracellular space. But when tissues that have entered the final state of injury and deterioration, the swollen dead tissues readily gave up its water after a similar spinning.

SUMMARY

Isolated mouse brain, kidney, and other tissues were incubated for from one to several days at $4^{\circ}C$ in isotonic solution containing varying mixtures of sucrose and NaCl (or Na_2SO_4 , LiCl, Li_2SO_4 , $MgSO_4$). The ischemic, cold-injured tissues swelled in proportion to the concentration of NaCl or LiCl. They swelled less in Na_2SO_4 and Li_2SO_4 and they swelled even less and/or shrank in $MgSO_4$ or sucrose. It was shown that in the presence of about 100 mM NaCl, the degree of swelling follows inversely the level of ATP present in the cells. The data were interpreted on the basis of the theory of cell swelling based on the association-induction hypothesis: NaCl and LiCl induced swelling in injured tissues was compared with KCl-induced swelling in normal tissues. Both were explained as the consequence of the dissociation of the volume-restraining effects of salt linkages among cell proteins. It is the depletion of ATP and the consequent increase of electron density or c-value of the fixed anionic groups that turns the ineffective NaCl (in causing swelling of normal tissues) into the fully effective agent for causing swelling of the injured tissue.

ACKNOWLEDGMENT

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LEGENDS

- Figure 1 - Theoretical curves of the adsorption energies (ordinate) of various cations on an oxyacid when the electron density (expressed as c-value) of the oxyacid group changes. Lower c-value (toward the left side of the abscissa) represents the equivalent of lower pK_a value and vice versa (from Ling, 1962).
- Figure 2 - Time course of weight change of isolated intact mouse kidney (A) and of mouse kidney cut into halves (sagittally) (B). Four sets of tissues were weighed repeatedly after light blotting on wetted filter paper. The bathing solution is a normal mammalian Ringer solution containing 118 M NaCl; its composition described in Table 1. Ordinate represents the relative volume, obtained by dividing the weight after a certain length of time (W_{fin}) by the initial fresh weight (W_{init}).
- Figure 3 - The relative weight of isolated mouse kidney, liver, and spleen following incubation for 1 day in mixtures of the two stock solutions (I and II) described in Table 1. All solutions are isotonic, but those to the left of abscissa have more sucrose while those to the right have more NaCl referred to in the abscissa as salt. The right hand-most experimental point of each of the 3 curves corresponds to that of a normal mammalian Ringer solution containing 118 M NaCl.
- Figure 4 - The relative weight of isolated mouse kidney following incubation for one day at 4° C in mixtures of sucrose-Ringer solution (Stock Solution I) and isotonic Ringer solution (Stock Solution II) or its variants containing instead of NaCl, LiCl, Li_2SO_4 and $MgSO_4$.

Figure 5 - The relative weights of isolated half mouse brain following incubation for two days at 4°C in mixtures of sucrose-Ringer (Stock Solution I) and either Stock Solution II containing 118 M NaCl or a modified Stock Solution II in which Na_2SO_4 has replaced the NaCl at isoosmotic strength (i.e., $2/3$ M of Na_2SO_4 replaced 1 M NaCl).

Figure 6 - The relative weights of isolated half mouse brain following incubation for four days at 4°C in mixture of sucrose-Ringer (Stock Solution I) and modified Stock Solution II in which the NaCl was replaced either by LiCl (1 mole for 1 mole) or Li_2SO_4 ($2/3$ M Li_2SO_4 for 1 M NaCl).

Figure 7 - The relation between the water contents of mouse brains and their ATP contents after the isolated half brains were incubated for various lengths of time in hours (indicated by number in brackets near each point). Each point represents average 4 or 3 determination \pm S.E.

Table 1 - Composition of the two Stock Solutions used in preparing the solution of varying salt and sucrose concentrations. Each stock solution was prepared in three separate components which were mixed just before use. GIB medium was obtained from General Biological in the form of sterile powder free of NaCl or KCl and was dissolved in distilled water before mixing with the antibiotics. Experimental solutions were prepared by mixing Stock Solutions I and II (or its modification in which the NaCl had been replaced by LiCl, Li_2SO_4 , MgSO_4 , etc.) in different proportions.

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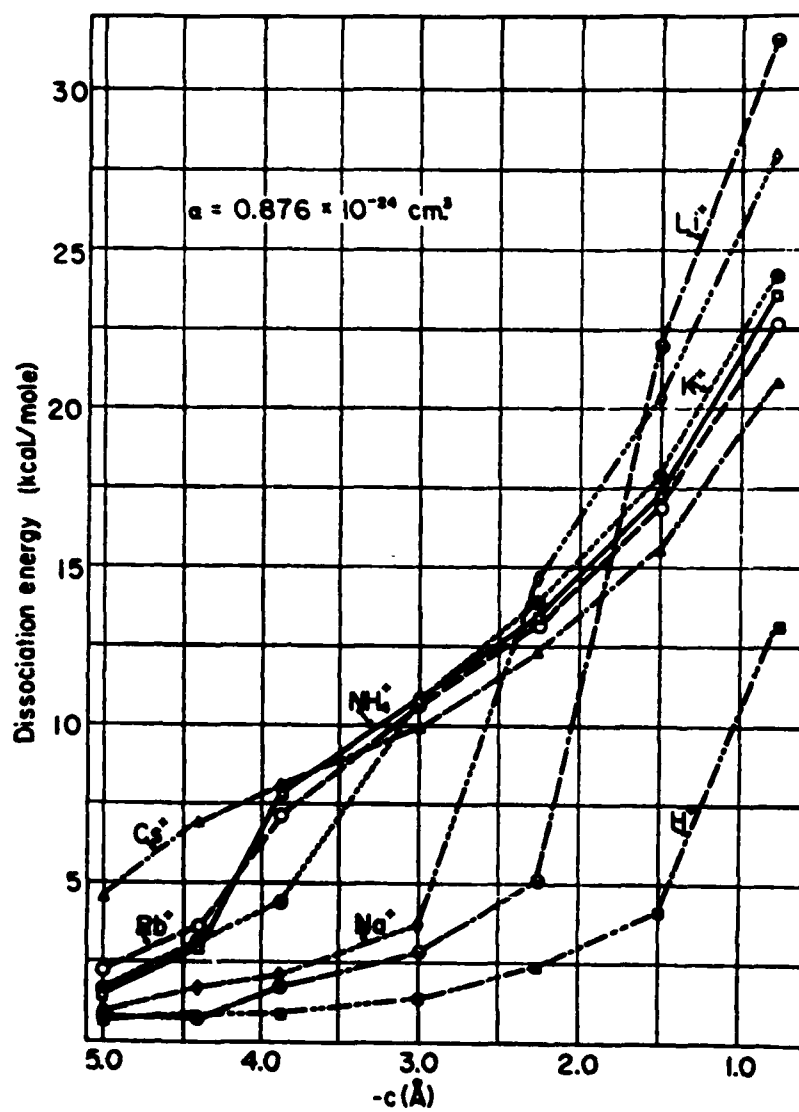


FIGURE 1

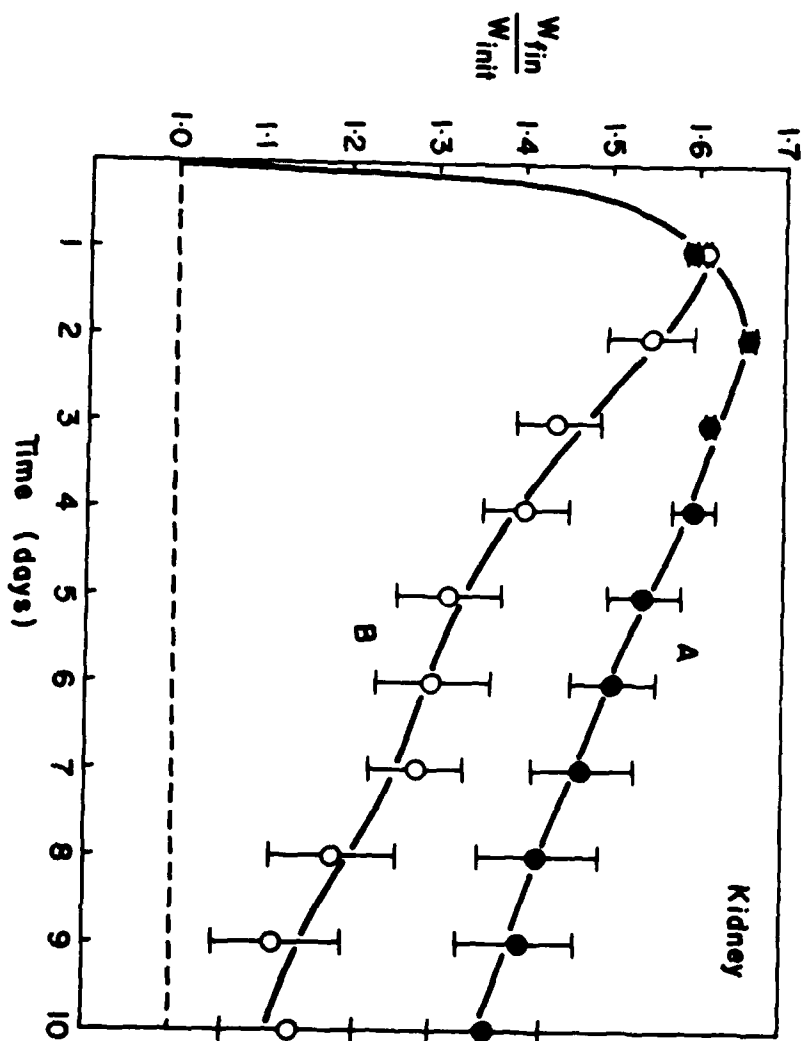


FIGURE 2

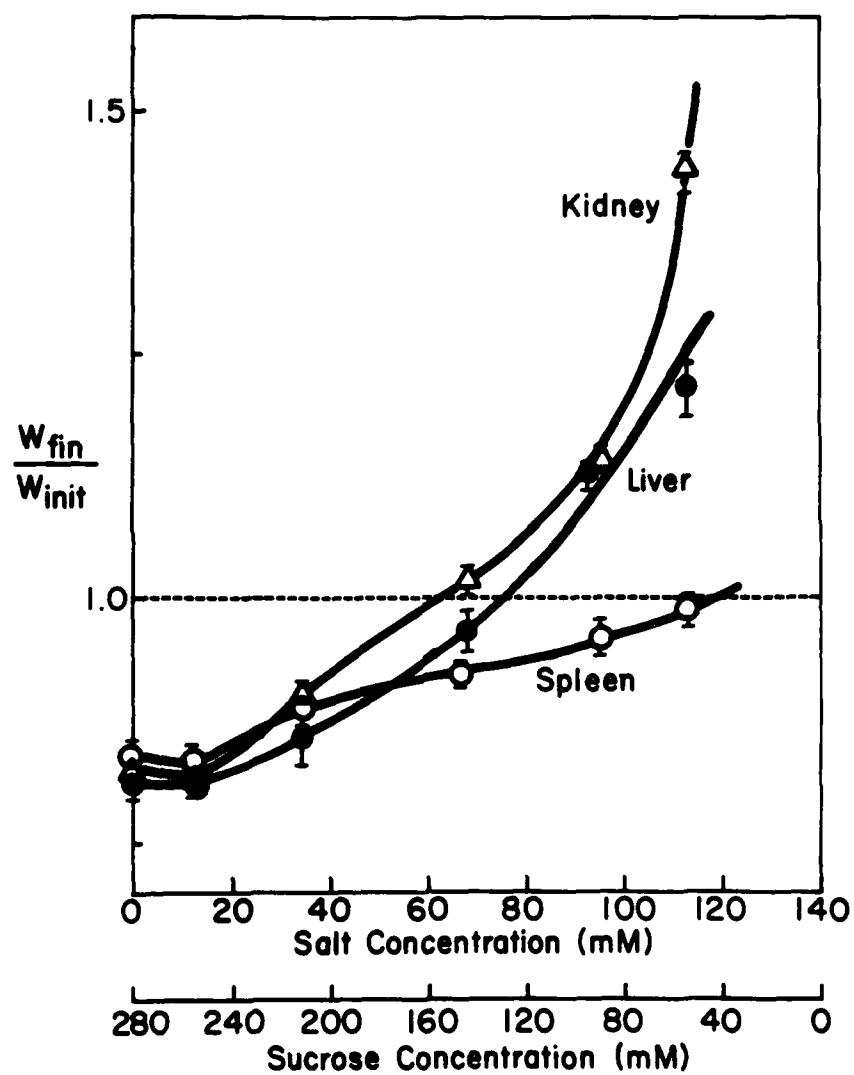


FIGURE 3

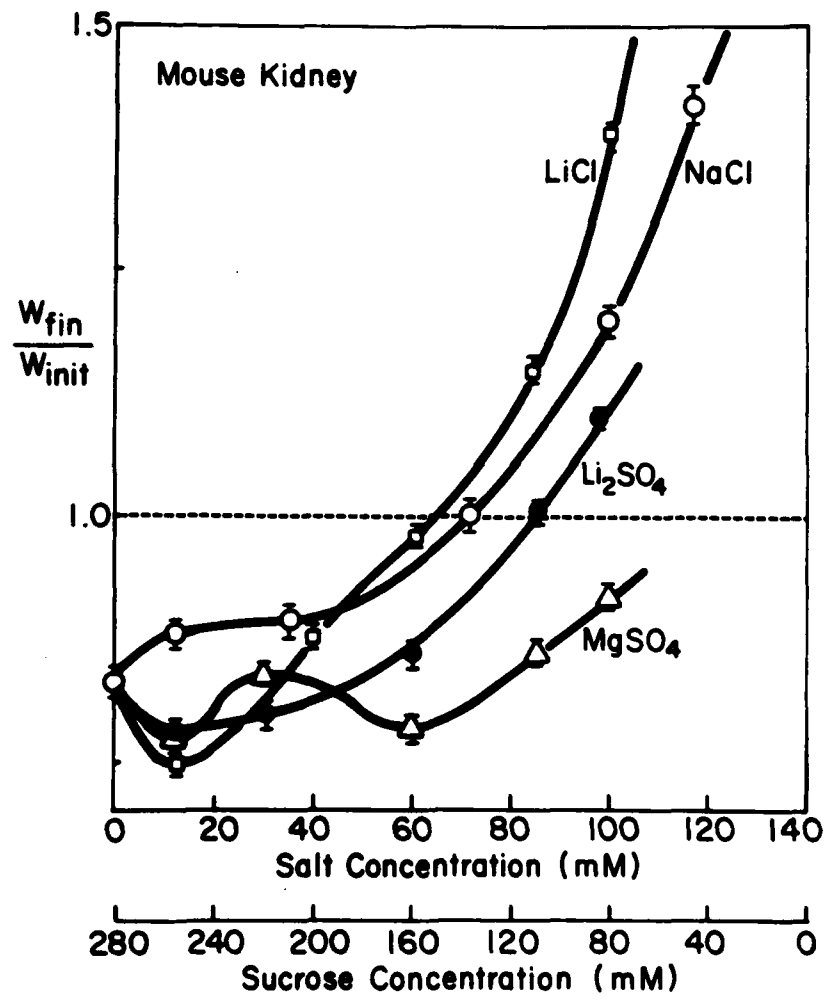


FIGURE 4

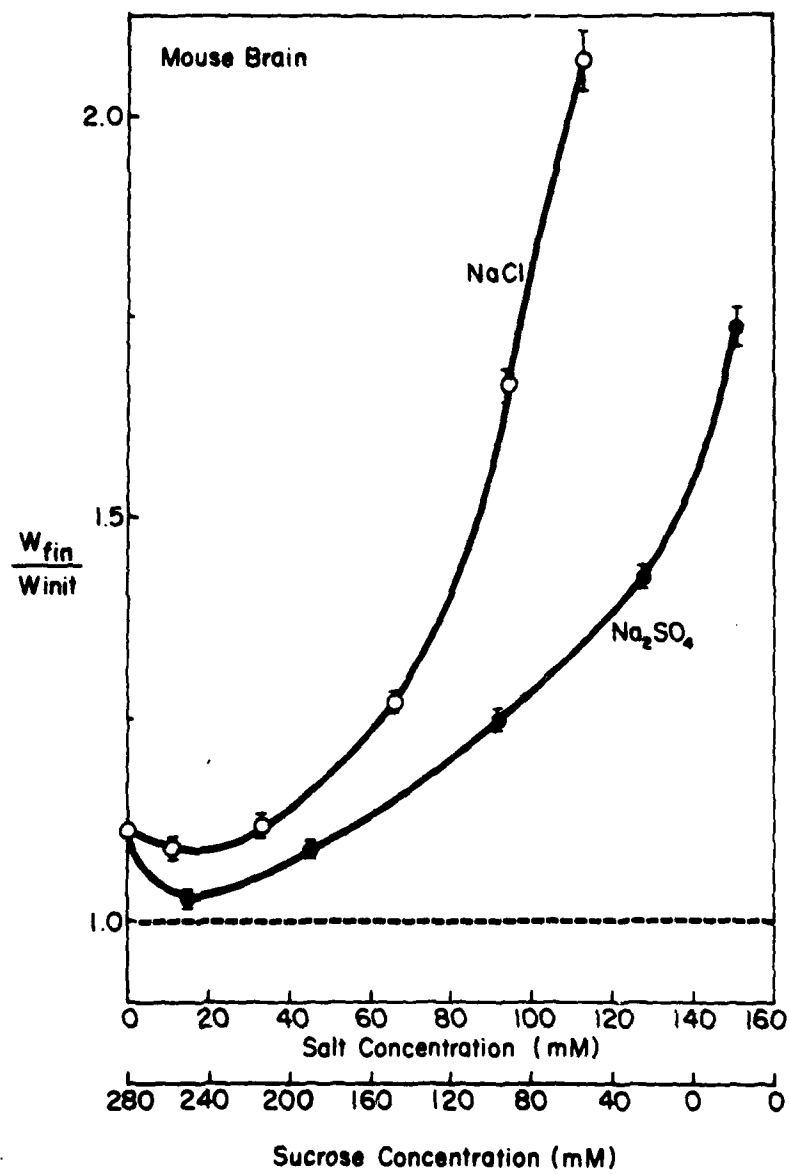


FIGURE 5

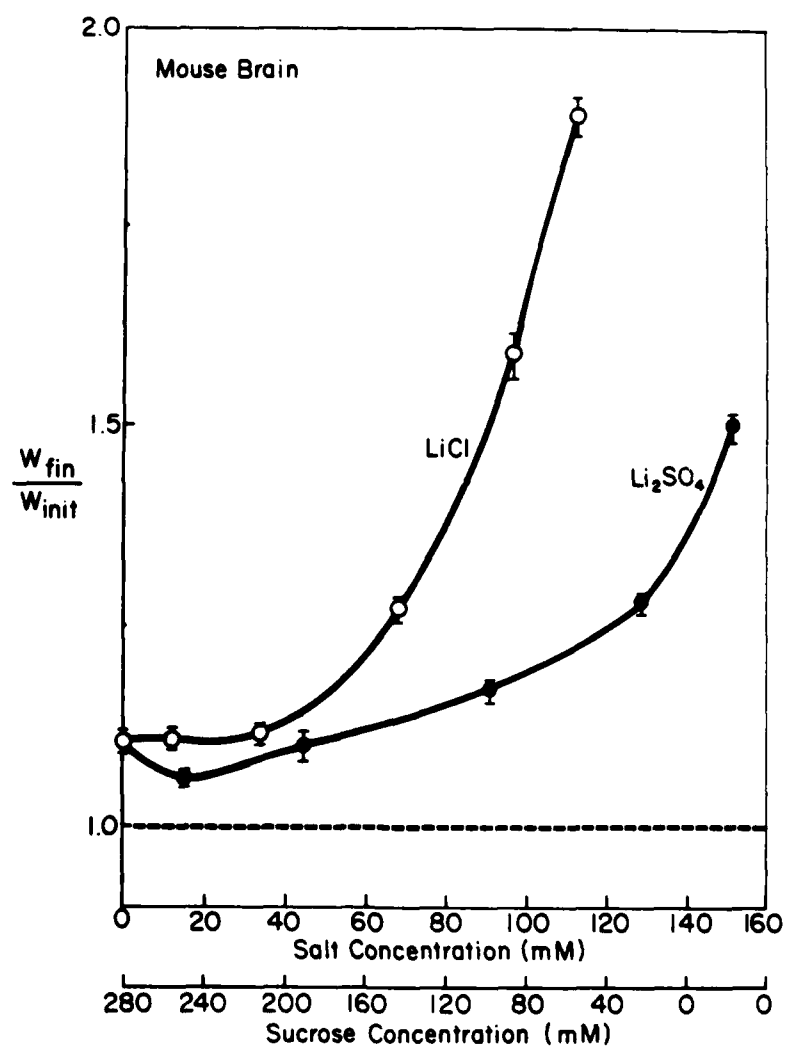


FIGURE 6

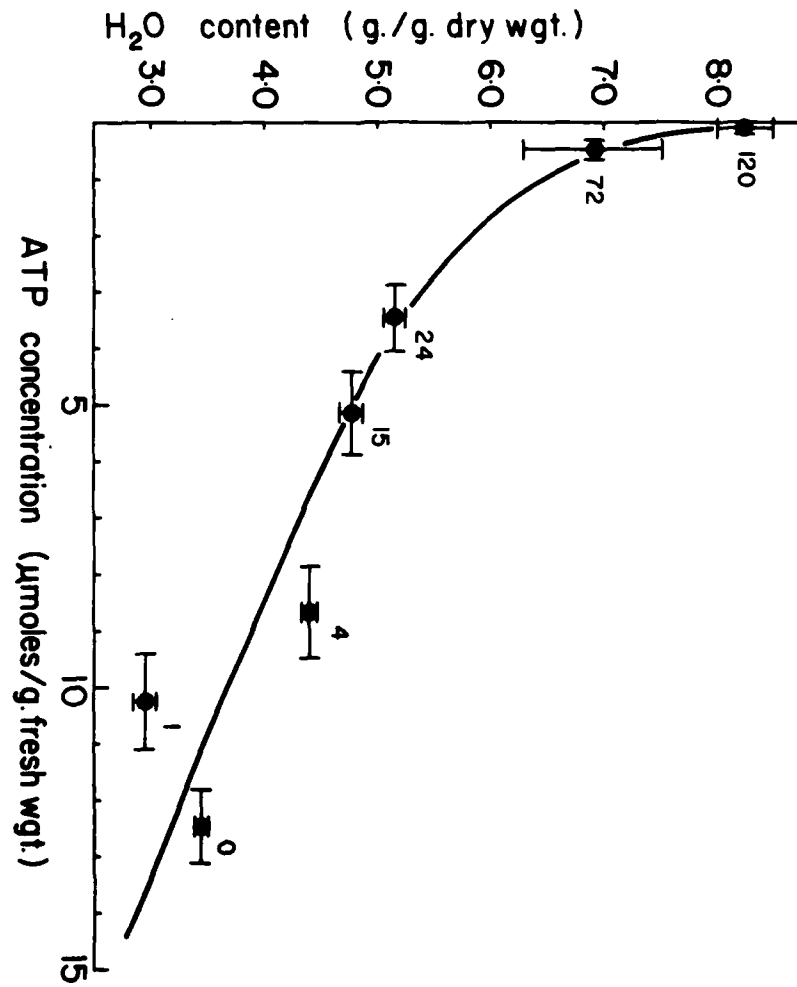


FIGURE 7

		<u>Stock Solution I</u>	<u>Stock Solution II</u>
	<u>Conc. (M)</u>	<u>Volume (ml)</u>	<u>Volume (ml)</u>
A	NaCl	5 X 0.154	26.76
	Sucrose	5 X 0.308	-
	KCl	0.154	27.96
	NaHCO ₃	0.154	4.30
	Choline bicarbonate	0.154	-
	NaH ₂ PO ₄	0.154	22.50
	H ₂ O	0.154	1.20
		83.8	83.8
B	MgCl ₂	0.11	1.2
	CaCl ₂	0.11	1.2
	Glucose	0.11	2.75
		0.308	2.75
		5.6	5.6
E	GIB medium (KCl- and NaCl-free)	15.4	15.4
	H ₂ O	10.0	10.0
	Penicillin (Na)	13.0 mg	13.0 mg
	Streptomycin	13.0 mg	13.0 mg

TABLE 1

STUDIES ON THE PHYSICAL STATE OF WATER IN LIVING CELLS AND
MODEL SYSTEMS. III. THE HIGH OSMOTIC ACTIVITIES OF AQUEOUS
SOLUTIONS OF GELATIN, POLYVINYLPYRROLIDONE AND POLY
(ETHYLENE OXIDE) AND THEIR RELATION TO THE REDUCED
SOLUBILITY FOR Na^+ , SUGARS, AND FREE AMINO ACIDS.

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19107

• Very high osmotic activities of concentrated aqueous solutions of gelatin, polyvinylpyrrolidone, and poly(ethylene oxide) were recorded. These observed values are far above those predictable from the molar concentrations of these polymers or those of aqueous solutions of native hemoglobin of equal concentrations. It was shown that these high osmotic activities are closely associated with the ability of the gelatin- or polymer-dominated water to exclude Na^+ salts, sucrose, and glycine. Both phenomena are interpreted as reflecting the polarization of multilayers of water by the polymers enhancing the H_2O to H_2O interaction and also reducing the translational and rotational motional freedom of the water.

INTRODUCTION

Gelatin, which is denatured collagen and a major component of glue, has long interested biologists and chemists. Thomas Graham used gelatin to represent a class of substances which he called colloids (Kollod , glue) (Graham¹). Katz,² Kunitz,³ Bungenberg de Jong and colleagues (Holleman et al⁴), Lloyd and Moran,⁵ and others discovered many important and unusual attributes of the water in the gelatin-water system. Recently by making use of a modification of the dialysis technique, which was also introduced by Graham (i.e., the equilibrium dialysis method), Ling and coworkers^{6,7} presented evidence that the unusual solvent properties of the gelatin-water system might arise from the extensive interaction of multilayers of water with the gelatin molecules. Their reasoning was as follows.

Due to the presence in the gelatin molecule of an abundance of glycine, proline, and hydroxyproline (Veis⁸), all well-known helix breakers (Chou and Fasman⁹), a major part of the gelatin polypeptide chain exists in an extended conformation and thus directly

exposed to the bulk-phase water. According to the association-induction hypothesis, it is the NH and CO groups of the extended and exposed polypeptide chains that polarize and orient multilayers of water and cause the change in water solvency (Ling¹⁰⁻¹²).

In harmony with this view, 13 globular proteins, including hemoglobin, which show no or very little effect on water solvency acquired the ability to do so when these proteins were exposed to urea or guanidine HCl (Ling et al^{6,7}). It was argued that native globular proteins affect water little or not at all because their NHCO groups are locked in α -helical or other macromolecular H-bonds.

Urea and guanidine HCl unravel the secondary structure of proteins, thereby exposing the bulk-phase water to the polarizing influence of the NHCO groups; denaturants like SDS and n-propanol that unravel only the tertiary structures had little or no effect. Additional support for the role of exposed polypeptide groups in reducing water solvency came from the studies of certain macroscopically electrically neutral polymers. Although these polymers lack the

NH groups of the extended polypeptide chains, they too possess oxygen atoms at regular distances apart, and like the oxygen atoms of the peptide CO groups, the distances between the nearest neighboring oxygen atoms are roughly equal to twice the diameters of water molecules (Ling et al⁶). As anticipated, they cause solute exclusion from the surrounding water. The most outstanding among these polymers are polyvinylpyrrolidone (PVP), poly(ethylene oxide) (PEO), and polyvinylmethylether (PVME).

Our interest in gelatin and these synthetic polymers lies in the belief that they may serve as a model for certain important cellular proteins which endow water in living cells with some of its unique characteristics. From the viewpoint of the association-induction (AI) hypothesis (Ling¹³⁻¹⁵) the ability of water dominated by gelatin, PEO, PVP, etc., partially to exclude Na⁺ and other solutes has great significance. It supports the view that the exclusion of these solutes in living cells may arise from a similar mechanism. That is, certain as yet unidentified matrix proteins (though actin, myosin, tubulin, and other cytoskeletal proteins are being considered as candidates) existing throughout the cell, may, like gelatin, urea-denatured proteins, PEO, PVP, etc., also exist in an extended conformation and in this state, polarize virtually all the intracellular water molecules. In the water thus polarized (in multilayers), the solubilities of small molecules and molecules that can fit into the multilayer dynamic structure remain normal (or even somewhat higher than normal as is known to be the case in living cells, Ling¹⁵ Ling et al¹⁶). For most molecules, due to enthalpic, entropic or both factors, the solubility decreases with increasing size and complexity of the solute involved (Size rule) (Ling^{10,12}; Ling and Sobel¹⁷). Among the solutes excluded are hydrated Na⁺, sugar, and glycine, which have also long been known to exist in much lower levels in living cells than in the surrounding media. A once popular theory, the membrane-pump theory, argued that these solutes are continually pumped out of the cells. However, extensive evidence now exists refuting this view (Ling^{11,13,14,15,18}; Ling and Negendank¹⁹). Additional evidence against the membrane-pump theory is provided by the adsorbed state of cell K⁺.

Not all solutes exist in low concentration in the cell. Some solutes accumulate in living cells at a level substantially higher than that found in the surrounding medium, as it is in the case of K⁺. In the AI hypothesis, the preferential accumulation of K⁺ involves selective adsorption of K⁺ on β - and γ -carboxyl groups of intracellular proteins (Ling^{13,20}; Ling and Ochsenfeld²¹). In voluntary muscles, these anionic groups are localized primarily in the A band and Z-line (Ling²²). Therefore, most of the muscle cell K⁺ is expected to be found in the A band and Z-line also. These predictions have been confirmed by investigators in West Germany, in Hungary, and in the USA. In this task, they used a total of four techniques: (i) autoradiography of air dried (Ling²²) and frozen fresh muscle cells (Edelman²³); (ii) direct EM visualization of electron dense Cs⁺ and Tl⁺ in frozen dried muscle cells after these ions had stoichiometrically and reversibly displaced the cell K⁺ (Edelmann²⁴); (iii) dispersive x-ray microprobe analysis (Edelmann²⁵ Tigyi et al²⁶); and (iv) laser mass-spectrometer microprobe analysis (Lamma) (Edelmann²⁷). Other experimental evidence showed that the K⁺, Cs⁺, and Tl⁺ localization is the result of specific one-ion-on-one-site close-contact adsorption (Ling^{10,22}). Since K⁺ is the major cation of the cells, its adsorption and hence osmotic inactivity leaves unanswered the question, "What keeps the cell interior in osmotic equilibrium with an isotonic Ringer solution containing 0.1 M of free Na⁺ and free Cl⁻?"

Since osmotic activity is an expression of the decrease of the activity of the water present, the question posed above can be restated as follows, "What component of the living cell causes the lowering of the activity of the bulk of cell water to match that of a Ringer solution, now that we know it cannot be free K⁺?" According to the association-induction hypothesis, this component is primarily the same "matrix proteins" mentioned above, which were postulated to lower the steady levels of Na⁺, sugars, and free amino acids in the cell water (Ling²⁸). If this idea is correct, we would expect that water dominated by PEO, PVP, and gelatin at a concentration high enough to lower the solvency of water for Na⁺, sugars, and free amino acids should exhibit osmotic activity higher than that calculated on the basis of the molar concentration of the polymers. This report describes results from experiments designed

MATERIALS AND METHODS

To measure the osmotic activity of polymer-water system, a Wescor Vapor Pressure Osmometer (Model 5100B, Wescor, Ind., Logan, Utah) was used. This small and versatile instrument measures the vapor pressure of the solution in a closed chamber by monitoring the dew-point temperature depression (which is a function of the vapor pressure) with a precision thermocouple hygrometer. While the instrument was designed originally for handling solutions of low viscosity, it was found suitable to measure osmotic activity of highly viscous solutions as most of the samples studied were. The main departure in the procedure used from the standard one was to deposit the sample in the sample holder first and to place the paper sample disc over the sample. Trials showed that this modification does not in any way adversely affect the results. Readings taken over a span of time yielded the same results. This verifying procedure was followed when new samples of different consistencies were measured.

Solutions (or gel) of three synthetic polymers and two proteins (gelatin and hemoglobin) were studied. The sources of these polymers were as follows: Polyvinylpyrrolidone (M.W. 360,000) (PVP-360), Lot 57c-0071 was from Sigma Chemical Co.; poly(ethylene oxide) (PEO) was a gift of Union Carbide. Gelatin, obtained from Eastman, was from pig skin (Lot A4-C, IEP 8.7, ash content 0.0340), and from calf skin (Lot B4B, IEP 4.7, ash content, 0.0290). Hemoglobin from bovine erythrocytes was also purchased from Sigma Chemical Co.; it existed as a mixture of methemoglobin and oxyhemoglobin.

We found that as a rule, samples of synthetic polymers as they were received from the suppliers contained very little ionic residues after ashing. On the other hand, gelatin, though of the highest quality obtainable commercially, did contain considerable ionic contaminants. To purify, dilute solutions (ca. 2%) of gelatin, hemoglobin as well as all the polymers were first prepared and then

exhaustively dialyzed against ion-free distilled water until ashes prepared from the dried samples of the polymer solution (600°C, 24 hrs. in a muffle furnace) yielded no measurable osmotic activities in the Wescor osmometer when dissolved in 10 mM HCl. The water contents of the dialyzed polymer solutions, while still in the dialysis sacs, were reduced in steps by either being placed in front of a fan in a cold room or packed in a dry dust-free silica gel (Davidson, mesh size 6-16). Great care was taken not to let the polymer dry unevenly. These methods yield preparations of homogeneous samples of polymer-water systems of widely varying water contents, which were assayed by oven drying at appropriate temperature (100°C for PVP, PVME, hemoglobin, and gelatin; 60°C in vacuo for PEO).

RESULTS

Figure 1 shows the osmotic activity of a solution of exhaustively dialyzed gelatin, where the osmotic activity is expressed in units of OsMolal and the gelatin concentration in percentage (W/V). For comparison, data from hemoglobin solutions are also presented. Included also in this figure are six experimental points from the osmolarity measurements of hemoglobin of Adair (Adair,²⁹ Adair and Robinson³⁰). Adair's data points in general agree with our own but they do not reach to as high a concentration as our own (up to 50%). Comparing the osmotic activity of gelatin with that of hemoglobin at equal protein concentrations one finds that the osmotic activity of gelatin is much higher especially at the higher concentration range. Neither the osmotic activity of gelatin nor that of hemoglobin is commensurate with the molar concentration of the proteins present. Thus a 50% hemoglobin solution is roughly $500 \cdot 6.7 \times 10^4 = 7.45 \text{ mM}$, while the osmotic activity measured corresponds to a concentration of 450 mM. The

$$\frac{\pi}{C_2} = RT \frac{V_1^0}{V_1} \left[M_2^{-1} + BC_2 + CC_2^2 + DC_2^3 + \dots \right] \quad (1)$$

where R, T have the usual meanings. V_1^0 is the volume per mole of pure solvent used; V_1 is the partial molar volume of the solvent in the polymer solutions. M_2 is the molecular weight of the macromolecule in units of g mol^{-1} . B, C, and D are the second, third, and fourth virial coefficients in units of mole l g^{-2} , $\text{mol l}^2 \text{g}^{-3}$, and $\text{mol l}^3 \text{g}^{-4}$ respectively. However, following tradition, the virial coefficients tabulated from these data are given in units of mol ml g^{-2} , etc. (Table I).

These plots show steep curvatures. Hence unusually large 3rd or even 4th virial coefficients are required to fit the data. Just how large these virial coefficients really are, is revealed by a comparison of the PVP and PEO curves with similar plots of hemoglobin in the same figures.

DISCUSSION

In answer to the main question raised in the Introduction, the observations presented in this communication show that the osmotic activities of the aqueous solutions of the two neutral polymers (PEO and PVP) and one charged protein (gelatin), are indeed much higher than that predicted by the molar concentration of the polymers present or the osmotic activity of the globular protein, hemoglobin, measured with the same instrument. These studies are in harmony with the theory that the osmotic pressure in living cells does not originate from free solutes like K^+ as was once widely believed but is primarily due to extended chains of some intracellular (matrix) proteins. However, before final acceptance of this conclusion, certain trivial causes for the observations must be considered.

Inadvertent oxidation reactions might give rise to, say, carboxyl groups and counterions which might increase the total osmotic activity beyond that of the originally neutral polymer. To test this possible trivial cause of the observed osmotic activity I analyzed the Na^+ contents of samples of PVP and PEO

after first equilibrating them in 0.1 NaCl and then exhaustively dialyzing the solution in distilled water which was made slightly alkaline with the addition of NaOH. The data revealed a total Na⁺ content amounting to a few micromolar in a 40% polymer solution. This level of contaminant is far too trivial in magnitude to make any significant difference to the data collected. A second possible source of error is the heterodisperse molecular weights of the polymers studied. However, the polymers had all been exhaustively dialyzed in dialysis tubings with a molecular cut-off point of about 12,000 daltons. Thus, even if the actual molecular weights of all the polymers studied were not as designated but were only 12,000, a 40% polymer solution would still be no more than $400 \times 12,000 = 30$ mM. This osmotic activity is far from the recorded osmotic activity of more than 1000 milliosmolar. Thus heterodisperse molecular weights could not make any significant contribution to the activities observed either.

Having eliminated contaminants and heterodisperse molecular weights as the causes of the high osmotic activity observed, I conclude that gelatin, PVP and PEO, which have been shown to have the power to reduce the solubility of water for Na⁺ salts, sugars, and free amino acids, do indeed also have powerful effects in reducing the activity of water in general and especially when the polymer reaches a certain high concentration. This pattern of behavior of the two synthetic polymers, PEO and PVP, and gelatin is shared to a minor degree by hemoglobin, a native globular protein.

The Structural and Concentration Requirements of the Osmotic Effect of Polymers. The minimal structural requirements for the polymer to have the pronounced water-activity-reducing effect are the same as those found for producing the reduction of solubility for Na⁺, sucrose, and glycine: the possession of oxygen atoms at regular intervals about two water diameters

apart, with these oxygen atoms freely exposed to the bulk phase water and not locked in α -helical or other intra- or inter-macromolecular H-bonds (Ling et al⁷).

The present findings thus provide new evidence that the unusual property of gelatin, and hence Graham's colloid, may reside in the powerful effect of its extended polymeric chains in reducing the activity of water in its surrounding medium.

Figure 1 clearly shows that at all concentration ranges, gelatin has much greater osmotic or water-activity-depressing effect than hemoglobin. A similar observation of the high osmotic effect was long ago noted by Kunitz³ who studied the osmotic pressure of gelatin up to a concentration of only 18%. Yet we have shown in our new data that it is when the gelatin reaches the critical concentration at about 45% that the effect becomes truly pronounced. This type of behavior reminds one of the sigmoid-shaped oxygen uptake curve of hemoglobin which is generally acknowledged to be due to cooperative interaction among the heme-sites on which oxygen molecules are complexed; the binding of one oxygen molecule enhances the affinity of other sites for more oxygen. In our present case, what one sees is that as the polymer concentration increases, its effects on the water activity also demonstrate characteristically cooperative behavior. Let us examine what could be the basis of this phenomenon, focusing our attention first on PEO.

Being simply repeating units of $(-\text{CH}_2\text{CH}_2-\text{O}-)_n$, this polymer has no side chains and the only seats of direct interaction with water are the oxygen atoms in the chain. Therefore the effect of increasing PEO concentration on water activity could only be due to a synergism between the water-activity-reducing effect produced by one oxygen atom on one chain and similar effects exerted by oxygen atoms on other chains when the average chain-to-chain distance decreases to a close enough value.

e gelatin, PVP and PEO all show similar sigmoid-shaped curves in their water-activity-reducing action and since the only H-bonding groups they share are the regularly alternately separated oxygen atoms on the backbone of these models taken together supports the view that certain ordered protein chains in living cells may be responsible for the reduction of water activity to match that in the external medium (water, plasma, etc.), which owe their activity-reducing effects to free Na^+

solution. Therefore, a comparison of the water-activity-reducing effect and the solvency reduction effects can be made legitimately. Closely parallel behaviors observed include sharp changes at certain polymer concentration and the relative effectiveness of both sets of effects among the three polymers studied in the rank order: gelatin $< \text{PVP} < \text{PEO}$.

One of the reasons previously given for the greater solvency-reducing effect of PEO than the two other polymers is that due to its extreme simplicity of structure and the lack of any side chains, it cannot form hydrophobic bonds or interaction H-bonds as is possible in the case of gelatin (Ling et al⁶).

Relationship Between Water-Activity Reduction and Water-Solvency Reduction in Polymers and Proteins. Figure 6 plots the μ -value for Na^+ in varying concentrations of PVP, and PEO. Note that in a 40% solution, the μ -value for Na^+ has dropped to about 0.1. That is, at least 90% of the activity has been so profoundly affected by the polymer that it has lost all its solubility for Na^+ . In fact, this exclusion of Na^+ would hardly be absolute for any volume of water. Thus it is more reasonable to suppose that virtually all the activity has been profoundly affected by PEO solvency.

Speaking of the PEO, PVP, and PVP concentrations represented here in Figure 6 cannot be directly compared with the data in Figures 1 and 3. While the activity measurements shown in Figures 1 and 3 were made on the basis of the polymer systems containing no Na^+ the data shown in Figure 6 were for polymer-water-systems containing Na^+ . Indeed it was by varying the concentration of the Na^+ citrate that the activity of the system, enclosed in dialysis tubing, different water contents were the subject of swelling and shrinkage of the polymer-water system in dialysis (as described by Ling²⁸). However, the comparison concerns 40% PEO. At this concentration the Na^+ citrate polymer-water system is only slightly different from the surrounding Na^+ -citrate

The parallel behavior between the water-activity-reducing effect and the solvency-reducing effect of these oxygen-containing polymers and proteins is to be expected from the proposed mechanism for both phenomena. The water-activity-reducing effect is seen as the consequence of the polarization and immobilization by the propagated polarization emanating from the oxygen atoms and the consequent reduction of, in particular, the translational and rotational "partition functions" of the water molecules in statistical mechanical terms (Ling^{10,11,12,34,35}; Ling et al³⁶). Such a reduction of translational and rotational partition functions lowers the vapor pressure of the water, which was in fact what we actually observed with the Wescor vapor-pressure osmometer. At this point, it is most gratifying to learn that the recent quasi-elastic neutron scattering studies of Rorschach, et al³⁷, not only have demonstrated reduction of translational and rotational freedom of water in living cells of brine shrimp cysts but also in PEO-dominated water (Rorschach³⁸).

The solvency-reducing effect has also been explained in terms of a translational but primarily rotational partition function reduction not of the water molecules themselves but of the large, complex solute molecules or hydrated ions (Ling^{10,12}). However, reduction of motional freedom of these solutes is the

consequence of the reduction of the motional freedom of the solvent water molecules, in the same sense that the loss of motional freedom of a butterfly when it is caught on a spider web is the consequence of the attachment to the immobilized elements of the spider web.

Quantitative Consideration of the Postulated Matrix Proteins in Living Cells. The concentration of polymers needed to produce a pronounced effect on water activity and on solvency is as a rule quite high (e.g., 40-50%). Can this system be compared with that of the living cells? The answer is yes for three reasons.

First, the required osmotic activity of most living cells falls in the range of 0.2 to 0.3 OsMolal. One does not require a 40-50% PEO solution to produce this level of osmotic activity; a 20% PEO or 25% PVP or gelatin can do quite well.

Second, according to the polarized multi-layer hypothesis of cell water, the maximum effect on water polarization, solvency reduction and water-activity reduction occurs when the "matrix protein" chains are fully extended and are highly ordered as in the highly organized living cell interior. All the model systems studied are obviously far from this situation, being more like a randomly tangled mass. Testing the expected effect of ordering these disordered chains on lowering ρ -values, Ling et al.^{6,7} found that the ρ_{Na} for the PVP-water system did indeed decrease with stirring, which tends to line up the linear chains. These results are in full accord with the earlier report of Woessner and Snowden³⁹ who produced NMR evidence for increased water structuring as a result of the stirring of another polymer-water system, a solution of Kelzan®, a bacterial polysaccharide.

The third point concerns the relation between protein contents and distances between protein chains where cell water is found. Even though some cells (e.g., human erythrocytes) contain as much as 40% protein, most living cells contain 20 to 30% protein. Furthermore, only a part of this can be the matrix protein. The question is then, if

the postulated matrix chains are indeed fully extended, how would the content of these proteins affect the number of water molecules found between the nearest neighboring chains? Would there be enough matrix proteins effectively to control the properties of the bulk phase water? A simple calculation goes a long way toward answering these questions. Thus if one liter of cells contains n grams of matrix proteins, one can use an average amino acid residue weight of 112 (see Ling¹¹, p. 48), an Avogadro's number of 6.06×10^{23} , and a peptide linkage length of 3.5×10^{-8} cm, to find that the total length of the fully extended polypeptide chains of the matrix proteins equals $(n/112) \times 6.06 \times 10^{23} \times 3.5 \times 10^{-8} = 1.89 \times 10^{14} \times n$ cm. Cut into 10 cm long filaments, these filaments, uniformly distributed, in a $10 \times 10 \times 10$ cm cube, would be $\sqrt{1.89 \times 10^{13} \times n}$ or $4.34 \times 10^6 \times n$ filaments to each side. The distance between each nearest neighboring filament, would be $10 / (4.34 \times 10^6 \times n) = 2.30 \times 10^{-6} / n$ cm. Figure 7 shows a plot of the percentage of matrix proteins in a cell against the distance (right ordinate) and the number of water molecules between each pair of nearest neighboring chains (left ordinate), assuming a diameter of 3 Å for each water molecule.

What this figure demonstrates is that between the wide range of matrix protein contents from 5% to 80%, an amazingly modest change in the number of water molecules between the protein filaments occurs. Indeed, even at a matrix protein concentration as low as 5%, there are no more than 10 water molecules between a pair of nearest neighboring chains. This is a concentration that one can reasonably expect of the postulated matrix proteins, especially if one recalls that other globular proteins not directly participating in polarizing the cell water must take up space and would thus help to reduce the average chain-to-chain distance between the matrix proteins to below 10 water molecules. On the other hand, if one or more of the other non-matrix proteins in the cell resembles hemoglobin, the data of Figure 1 suggests that it too will contribute, though to a much smaller extent, to the water-activity reduction essential for the maintenance of normal cell volume.

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FIGURE 1. Osmolality of gelatin and hemoglobin at varying concentrations. Osmolality is given in Osmolal concentration. Protein concentrations are in C_r (wt. wt). Each point is the average of at least 4 independent determinations and the distance between horizontal bars are twice the standard errors. Six extra points on the hemoglobin curves shown as Δ 's are taken from Adair's data (see text).

FIGURE 2. Osmolality of polyvinylpyrrolidone (PVP) solution at varying concentrations. Details are the same as in Figure 1.

FIGURE 3. Osmolality of poly(ethylene oxide) (PEO) solution at varying concentrations. Details are the same as in Figure 1.

FIGURE 4. Plot of π/C against C in PVP solutions. Osmotic pressure, π , is in units of cm of H_2O C in grams of polymer per liter. Data are the same as in Figure 2, except that standard error bars are not represented. The solid line going through the experimental points are based on Equation 1. Values of virial coefficients chosen to fit the data are given in Table I. For comparison the hemoglobin data of our own and from Adair are also shown.

FIGURE 5. π/C vs. C plots of PEO solutions. Details are similar to those described in Figure 4. Value of virial coefficients are those given in Table I. For comparison hemoglobin data of our own and from Adair are also shown.

FIGURE 6. Plots of apparent equilibrium distribution coefficients of Na^+ salts (p -value) of polymer water system against polymer concentration given as C_c (w/w). (from Ling and Ochsenfeld⁴⁰).

FIGURE 7. The theoretical distances both in Ångstrom units (right ordinate) and in number of water molecules between nearest neighboring (fully extended) protein chains in hypothetical case when the entire protein contents (abscissa, in C_c (w/w)) are in the fully extended state and occupy no space.

TABLE I. Virial coefficients from the osmotic properties of aqueous systems of gelatin, PVP, and PEO.

TABLE I. Virial coefficients from the measured osmotic properties of aqueous systems of gelatin, PVP, and PEO.

	B (mol.ml.g ⁻²)	C (mol.ml. ² g ⁻³)	D (mol.ml. ³ g ⁻⁴)
PEO	5.54×10^{-2}	2.17×10^{-1}	—
PVP	1.25×10^{-3}	—	3.28×10^{-2}
Gelatin	3.29×10^{-2}	5.15×10^{-2}	—
Hemoglobin			1.5×10^{-3}

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The Molecular Mechanisms of Cellular Potentials

Gilbert N. Ling

I. INTRACELLULAR POTASSIUM: PUMPED OR ADSORBED?

Water, proteins, and potassium ions are the three most abundant components of the living cells. According to the membrane-pump theory, intracellular water and K^+ exist largely in the free state. This theory has provided the theoretical framework for a great number of brilliant achievements, including the theory of cellular potentials of Bernstein (1912), Hodgkin and Huxley (1952), and many others.

According to the alternative "bulk-phase theories" as championed by Moore and Roaf (1908), Fisher and Suer (1935), Gortner (1930), and others, a substantial part of the cells' K^+ and water may exist in a bound state. This view all but vanished in the early 1940s. The membrane-pump concept became almost universally accepted and taught. But not everyone was so convinced; among the doubters were Ernst (1963), Troshin (1966), and myself.

Having discovered that iodoacetate plus anoxia failed to slow down the rate of Na^+ efflux in frog muscle (Ling, 1951, 1952, 1962; which in the membrane-pump theory largely represents outward Na^+ pumping rate), a finding subsequently confirmed by Keynes, Conway and co-workers (Keynes and Maisel, 1954; Conway *et al.*, 1961), I reached the conclusion that the postulated Na pump would consume more energy than the cell commands. As an alternative, I suggested in 1951 and 1952 a new theory that includes a molecular mechanism for the selective accumulation of K^+ over Na^+ in living cells as well as in (sulfonate type) cation-exchange resins. This theory comprises four postulates: (1) fixed anionic (and cationic) sites exist throughout the living cell and not limited to the cell membrane as previously suggested (see below), (2) these fixed ionic sites belong primarily to proteins, e.g., β - and γ -carboxyl groups, (3) in living cells and some model systems there is a high degree of counter-ion (for example, K^+) association with fixed anionic sites, and (4) electrostatic adsorption on fixed anionic sites favors the smaller hydrated K^+ over Na^+ (Ling, 1951, 1952).

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A variety of experimental studies were carried out to test whether intracellular K^+ is free or "bound," including the vapor pressure measurement of Hill and co-workers (Hill, 1930; Hill and Kupalov, 1930), the measurement of K^+ mobility in nerve and muscle cells (Hodgkin and Keynes, 1953; Kushmerick and Podolsky, 1969), the measurement of intracellular K^+ activity with a K^+ -sensitive microelectrode (Hinke, 1961), the demonstration of an ability of pure natural cell membranes (red cell ghosts; Freedman, 1976), or synthetic membrane vesicles (phospholipid vesicles containing K - Na activated ATPase) selectively to accumulate K^+ or Na^+ (Hilden and Hokin, 1975; Goldin and Tong, 1974). These findings, in addition to the *apparent* contradiction (Berendsen and Edzes, 1973; Cooke and Kuntz, 1974) of early claims of demonstration of K^+ and Na^+ binding (Cope, 1965; Ling and Cope, 1969) as well as H_2O binding by NMR (Hazlewood *et al.*, 1969; Cope, 1969) led many scientists to the conclusion that the bulk of intracellular K^+ as well as water is in a free state.

More careful scrutiny of the evidence, however, has revealed that this conclusion was incorrect (a full in-depth review will be given in my forthcoming book (Ling, 1983), for a briefer and less complete one, see Ling and Negendank, 1980).

On the other hand, the pure membrane "vesicles," *par excellence*, i.e., the squid axon membrane sacs free of axoplasm do not pump K^+ or Na^+ (Ling and Negendank, 1980), whereas an effectively membraneless open-ended (EMOC) preparation of muscle cells does preferentially accumulate K^+ and extrude Na^+ (Ling, 1978a).

II. EVIDENCE THAT SUPPORTS BINDING OF INTRACELLULAR K^+

The most definitive experimental evidence that the bulk of intracellular K^+ exists in an adsorbed state has come in the last five years.

According to the Association-Induction (AI) Hypothesis, the bulk of intracellular K^+ is adsorbed on β - and γ -carboxyl groups of cellular proteins (Ling, 1952). Because in muscle over 60% of these anionic groups belong to myosin (Ling and Ochsenfeld, 1966), and myosin is found only in the A band of the muscle myofibrils (Hanson and Huxley, 1953), the theory predicts the localization of K^+ in the A bands. There is also belief that the sites that bind uranium in electron-microscope sections of tissues are also the β - and γ -carboxyl groups (Ling, 1977a). Accepting this view, the AI Hypothesis further predicts that if one can in some way visualize K^+ as it exists in the living cell, the pattern of its distribution would resemble a conventional electron-microscope picture, in which the darkly stained area (by uranium) would be where most K^+ is found. To test this hypothesis, I used autoradiography. Unfortunately, the two radioactive isotopes of K^+ were not suitable because one has too short a half life (^{42}K) and the other is much too expensive (^{40}K). For this reason, I settled for two "surrogates": ^{134}Cs and ^{201}Tl , both long-lived and inexpensive.

With a technique for preserving isolated frog muscle in room temperature for up to eight days (Ling and Bohr, 1969), most of the cell K^+ can be replaced physiologically and reversibly by ^{134}Cs -labelled Cs^+ or ^{201}Tl -labeled thallium (Tl^+ ; Ling, 1977b). Single frog muscle fibers, whose K^+ had been completely or largely replaced by labeled Cs^+ or Tl^+ , were isolated and rapidly air dried. After coating with photoemulsion and an exposure of about two weeks, the developed pictures appear as shown in Fig. 1, where the partial coverage of the emulsion allows identification of the lines along which silver granules congregate to be the A (dark) band.

This work was completely corroborated and extended by Edelmann (1980a), who used frozen (rather than dried) muscle fibers loaded with, for example, ^{134}Cs -labeled Cs^+ , carried out the exposure in liquid nitrogen and separated the emulsion film from the underlying

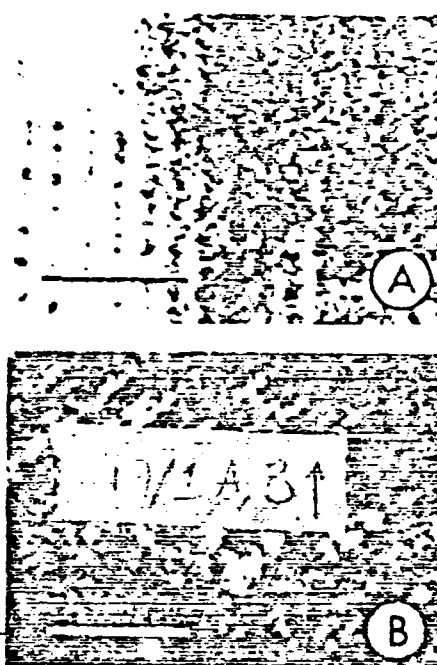


Figure 1. Autoradiographs of frog muscle fibers. Single ^{134}Cs -loaded fiber partially covered with photographic emulsion (From Ling, 1977a, by permission of *Physiol. Chem. Phys.*).

muscle fiber before taking light-microscopic or electron-microscopic pictures. Besides autoradiography, Edelmann carried out no less than three additional types of experimental testing of the predictions of the A1 Hypothesis, including transmission electron microscopy. In this work, Edelmann (1977) first replaced electron-light K^+ (at. wt. 39) in frog muscle cells with electron dense, Cs^+ (at. wt. 133) and Tl^+ (at. wt. 204). These Cs^+ - or Tl^+ -loaded muscles were then frozen dried, and dry cut. With neither chemical fixation nor staining, he obtained electron-micrograph plates (Figs. 2B and C) that reveal a pattern of distribution of these K^+ -surrogates strikingly similar to the glutaraldehyde-fixed and uranium-stained preparation (Fig. 2A). When the sections were exposed to water (Fig. 2E) and in normal " K^+ -loaded" muscle (Fig. 2F) only faintly dark areas show.

Edelmann then used another method to test the hypothesis (Edelmann, 1978), dispersive x-ray microprobe analysis. In this, an electron beam was focused on either the A band or I band of Cs^+ - or Tl^+ -loaded as well as normal " K^+ -loaded" muscle thin sections. The x-ray spectrum revealed that the concentrations of all three elements were higher in the A band than in the I band. This work has been fully confirmed by Trombitas and Tigyi-Sebes (1979), who demonstrated much higher K^+ concentration in the A bands than in the I bands of isolated myofibrils of honeybee thorax muscle.

The observations described above have established that in voluntary muscle K^+ is localized in distribution. Other evidence shows that the accumulated K^+ can be displaced by other univalent cations; the effectiveness varies greatly with different ions that have the same long-range attributes (that is, univalency) but differ by short-range attributes only. The fact that this ion-specific effect is fully preserved in muscle cells whose membrane (plus postulated pumps) were made functionally ineffective (EMOC preparation; see Ling, 1977b) suggests that the localized K^+ is specifically adsorbed, one K^+ to one anionic site. Additional evidence of the *adsorbed* state of K^+ and Ca^{++} distribution in the A band is derived from another new technique Edelmann developed.

Edelmann's (1980b) fourth effort to test the association-induction hypothesis was even

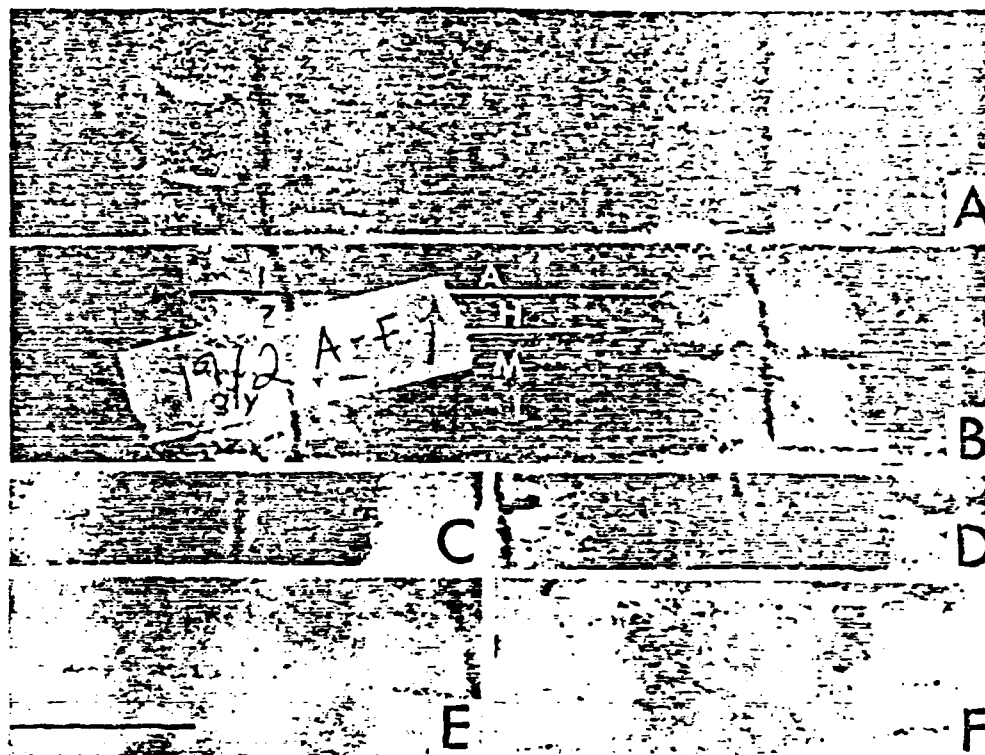


Figure 2. Electron micrographs of frog sartorius muscle. (A) Muscle fixed in glutaraldehyde only and stained with uranium by conventional procedure. (B) EM of section of freeze-dried Cs^+ -loaded muscle, without chemical fixation or staining. (C) Tl^+ -loaded muscle without chemical fixation or staining. (D) Same as (C) after exposure of section to moist air, which causes the hitherto even distribution of thallium to form granular deposits in the A band. (E) Section of central portion of (B) after leaching in distilled water. (F) Normal "K+-loaded" muscle. (From Edelmann, 1977, by permission of *Physiol. Chem. Phys.*).

more striking. He used the new technology called laser-microprobe mass spectrometric analysis (LAMMA). In this, he used freeze-dried and dry cut regular normal frog muscle cells. The thin sections obtained were dipped in a solution containing 50 mM K^+ , 50 mM Na^+ , and 10 mM Cs^+ . Freed of adhering fluid, the A band was vaporized by a focused laser beam and the vaporized atoms analyzed quantitatively. Peak heights, when compared to a control gelatin film containing known quantities of 50 mM K^+ , 50 mM Na^+ , and 10 mM Cs^+ , revealed preferential uptake of K^+ and Cs^+ over Na^+ . Here, there is no question of membrane or membrane pumps, as we are dealing with a thin section less than $1\mu\text{m}$ thick from a single muscle cell $60\mu\text{m}$ in diameter. Yet selective K^+ and Cs^+ over Na^+ adsorption can be demonstrated *in vitro*, yielding convincing evidence for a fundamental issue of century-old debate. The bulk of intracellular K^+ is adsorbed on anionic protein sites.

III. OSMOTIC ACTIVITY

The establishment of the adsorbed state of the major cation, K^+ , demands a "new" source of osmotic activity inside the cell to balance that of free Na^+ in the external medium

(Ling, 1980b). Remembering that osmotic activity in fact is a measure of the decrease of water activity, this "new" source, in the AI hypothesis, is the third major component of the cells, the proteins. More specifically, it is the extended polypeptide chain of a "matrix protein" postulated to exist throughout the whole cell in all living cells. Although, not yet clearly established, actin, tubulin, and other cytoskeletal proteins are likely candidates, though in the AI hypothesis, they must exist, in the resting living cells, in a finer state of dispersion than those usually seen in electronmicroscopic pictures.

The matrix protein or proteins polarize in multilayers and hence reduce the osmotic activity of the bulk of cell water amounting to, on an average, about ten layers of water molecules between two chains. It is the reduced solubility of water in this state for large hydrated ions, e.g., Na^+ , and molecules, e.g., sugar and amino acids, that functions to maintain the low level of these solutes in muscle, nerve, and other cells. Since there are no other osmotically active molecules or ions in the cell which match in concentration that of K^+ , the establishment of the adsorbed state of K^+ adds an important reason for the polarized multilayer theory of cell water. (For recent experimental evidence supporting this view, see Ling *et al.*, 1980a,b; Ling, 1981a).

One major concept of the association-induction hypothesis, a high degree of association between the major components of the cell (proteins, water, and K^+), is now reasonably well established by the experiments described above. The second major concept is yet far from being established, it concerns the control of coherent behavior through the propagated short-range inductive effect, or more completely the indirect *F*-effect, where the *F*-effect refers to the combined inductive or *I*-effects mediated through intervening atoms and *D*-effect mediated through space. A propagated *F*-effect is called an indirect *F*-effect (Ling, 1962). This provides a possible mechanistic basis for what later Monod *et al.* (1965) called "allosteric" effect (Ling, 1962, 1977d, 1981b). In the AI hypothesis this inductive effect lends coherence to the associated protein- K^+ -water system as an autocoperative assembly by providing a mechanism for near-neighbor interactions among adsorption sites for ions as well as H_2O . Just as important, the inductive effect provides the theoretical basis for the control of many binding sites for K^+ , H_2O and other molecules on the protein chains by a small number of "cardinal adsorbents," including ATP and Ca^{++} which adsorb on specific controlling sites of the proteins, called "cardinal sites." Cardinal sites include what are commonly known as receptor sites (Ling, 1977d, 1981b).

IV. SOLUTE DISTRIBUTION

A general equation for solute distribution in living cells and model systems was introduced in 1965 (Ling, 1965). Choosing K^+ (and Na^+) as examples, and assuming the existence of only one type of adsorption site, the equation reads:

$$[\text{K}^+]_{\text{cell}} = \alpha_{\text{K}} [\text{K}^+]_{\text{ex}} + \frac{[K^+]_{\text{ex}}}{2} \left(1 + \frac{[K^+]_{\text{ex}} \cdot K_{\text{Na-K}}^{\infty} - 1}{\sqrt{\left(\frac{[K^+]_{\text{ex}}}{[Na^+]_{\text{ex}}} K_{\text{Na-K}}^{\infty} - 1 \right)^2 + 4 \frac{[K^+]_{\text{ex}}}{[Na^+]_{\text{ex}}} K_{\text{Na-K}}^{\infty} \exp\left(\frac{\gamma}{RT}\right)}} \right) \quad (1)$$

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The first term on the right-hand side of Eq. (1) represents free K^+ in the cell water, and the second term represents adsorbed K^+ . The concentration of free K^+ in the cell depends on α , the water content, $[K^+]_{ex}$, the external K^+ concentration, and q_K , the (average) equilibrium distribution coefficient of K^+ in the cell water. The value of q is near unity for small molecules and molecules that can fit in the polarized water lattice but are low for hydrated ions ($q_{Na} \approx 0.1$; $q_K \approx 0.3$) and other larger molecules (Ling, 1977c; Ling *et al.*, 1980a,b; Negendank and Shaller, 1979). The term representing adsorbed K^+ is an adsorption isotherm that was introduced in 1964 (Ling, 1964). Here $[N]$ is the concentration of the adsorption sites for K^+ in moles per kilogram of fresh cells, K_{Na-K}^0 is the intrinsic equilibrium constant for $Na \rightarrow K$ exchange, $-\gamma/2$ is the nearest neighbor interaction energy between K^+ and Na^+ (for more details, see Ling, 1970, 1980a; Ling and Bohr,

1970). When $-\gamma/2$ is zero, the isotherm becomes in essence the usual Langmuir type.

However, when $-\gamma/2$ is larger than zero, the isotherm is autocoperative. The equilibrium concentration of K^+ adsorbed ($[K^+]_{ad}$) by the cells when plotted against increasing external K^+ concentration (at a constant $[Na^+]_{ex}$) is S-shaped or sigmoid, showing lower increment of uptake per unit increment of external K^+ concentration at low external K^+ concentration than at a higher external concentration. When the same data are plotted in a double logarithmic plot, i.e., when $\log [K^+]_{ad}$ or $\log ([K^+]_{ad}/[Na^+]_{ex})$ is plotted against either $\log [K^+]_{ex}$ (at constant $[Na^+]_{ex}$) or $\log ([K^+]_{ex}/[Na^+]_{ex})$, the slope at the locus where $[K^+]_{ad}$ equals $[Na^+]_{ad}$ is greater than unity. Indeed, the straight line with this slope and passing through the locus of equal K^+ and Na^+ occupancy is one described by the equation introduced by A. V. Hill (1910) to represent the oxygen uptake of hemoglobin. The slope of this double-log plot is the Hill's coefficient n , n , so far an empirical parameter, has long been suspected to be related to cooperativity. It is, in fact, equal to $\exp\left(-\frac{\gamma}{2RT}\right)$ where R and T have the usual meanings (Ling, 1964, 1980a).

The remarkable feature of an autocoperative adsorption is its "all-or-none" nature. That is, with a minor change in the ratio of K^+ and Na^+ concentration in the medium, the adsorption shifts from all K^+ to all Na^+ , as diagrammatically illustrated in Fig. 3A, where the i th and j th solute may represent Na^+ and K^+ respectively.

Experimental studies showed that in a variety of living cells, the K^+ and Na^+ distribution follows Eq. (1) well. These include frog muscles (Ling, 1966; Ling and Bohr, 1970), rabbit uterine muscle (Jones, 1970), canine carotid arteries (Jones, 1973), guinea pig taenia coli (Karremann, 1973; Gulati, 1973), and human lymphocytes (Negendank and Shaller, 1979). Furthermore, in agreement with the association-induction hypothesis, a number of agents including drugs such as ouabain and ions such as Ca^{++} , which collectively are referred to as cardinal adsorbents, act on the K^+ and Na^+ distribution by changing primarily K_{Na-K}^0 as diagrammatically illustrated in Fig. 3B. Thus, exposure of frog muscle to a low concentration of ouabain ($3.26 \times 10^{-7} M$) causes virtually all intracellular K^+ , to be replaced stoichiometrically by Na^+ by merely changing the value of K_{Na-K}^0 from 200 to about 10, as shown in Fig. 4. The fact that K^+ is adsorbed to begin with and that the ouabain effect is entirely intact in an EMOC preparation (Ling, 1978a) shows that ouabain does not act on membrane-pumping mechanism, but diminishes the selectivity of the adsorption sites of K^+ over Na^+ . Ca^{++} , on the other hand, acts to preserve the high K_{Na-K}^0 value, so that removal of Ca^{++} then acts like ouabain (Jones, 1973).

How a small number of bound Ca ions or ouabain molecules can affect many more K^+ and Na^+ adsorption sites is a subject of central interest in the AI hypothesis. As I have

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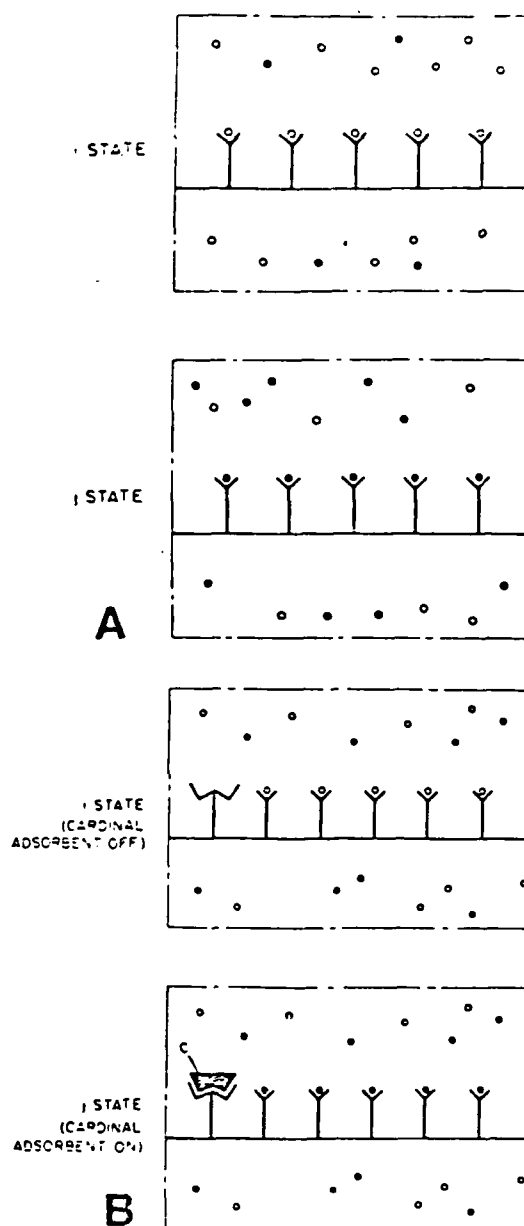


Figure 3. (A) Cooperative shifts between *i* and *j* states due to a change in the relative concentration of the *i* and *j* solutes in the environment. (B) Cooperative shifts between *i* and *j* states due to adsorption-desorption of cardinal adsorbent in an environment with unchanging *i* and *j* concentrations. (From Ling, 1977d, by permission of *Mol. Cell Biochem.*).

already mentioned, the propagated inductive effect or indirect *F*-effect offers the basic information- and energy-transmitting medium. I shall now review the mechanism of shift in $K_N^+ - K$ as a result of the propagated inductive effect.

The facts that sulfonate type of ion exchange resin with low pK_a selects K^+ over Na^+ and phosphoric and carboxy types of ion exchange resin with high pK_a values select Na^+ over K^+ (Bregman, 1953), the theory of Teunissen and Bungenberg de Jong (1938) that similar rank order of selectivity changes reflect differences of the field strength of the anionic groups, as well as the work of Eisenman *et al.* (1957; see also Ling, 1960) provided the stimulation to extend the earlier (1952), more restrictive model of selective adsorption of

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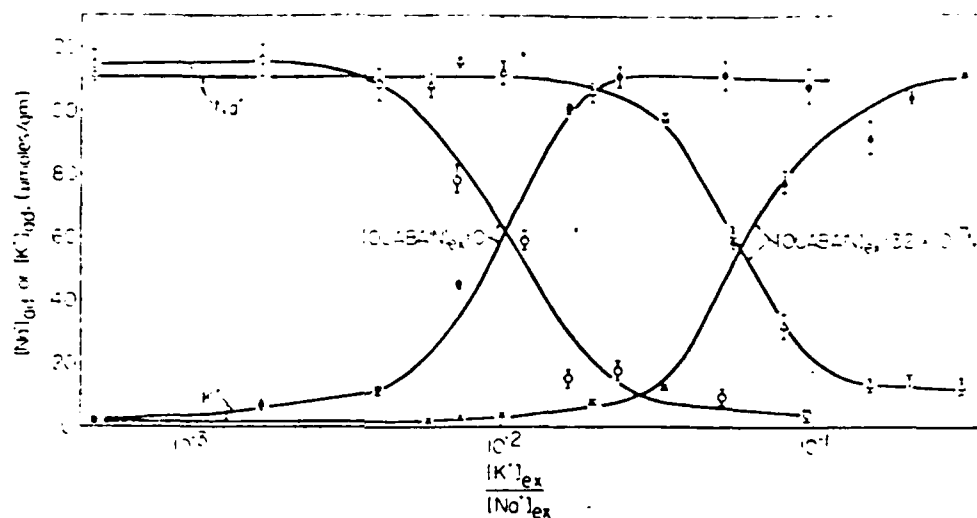


Figure 4. Effect of ouabain (3.2×10^{-7} M) on the equilibrium distribution of K^+ and Na^+ ion. Curves with open (Na^+) and filled (K^+) circles were equilibrium-distribution data from muscles not treated with ouabain. The point of intersection gives a K^+_{out}/K^+_{ex} of 100. In muscles treated with ouabain (3.2×10^{-7} M), K^+_{out}/K^+_{ex} has shifted to 21.7. (From Ling and Behr, 1971, by permission of *Physiol. Chem. Phys.*).

K^+ over Na^+ to a more general model in which selectivity of K^+ and Na^+ as well as other monovalent ions are variable (Ling, 1957, 1960). Indeed, in this microscopic model, the selectivity of K^+ and Na^+ varies with a small change in the electron density of an anionic oxygen atom, measured as a c -value. Rigorously defined elsewhere (Ling, 1962, p. 58), the c -value may be more simply described as a way to quantitatively simulate the aggregate effects of the remaining atoms of an oxyacid on the interaction of a hypothetical, prototype, singly-charged oxygen atom with a cation, as a displacement (in Angstrom units) of the unit electric charge on the oxygen atom from its original prototype location at the center of the oxygen atom. Thus, if the aggregate effect produces an overall displacement of electrons in the system toward the oxyacid oxygen, it can be exactly matched by a specific displacement of the unit charge toward the cation, represented as a positive c -value, e.g., $+1.0 \text{ \AA}$. On the other hand, if the aggregate effect is to produce the opposite effect, it would be represented as a negative c -value, e.g., -1.0 \AA . Thus, high c -value corresponds to high pK_a as in acetic acid, and low c -value corresponds to a low pK_a as in trichloroacetic acid.

In this theory, it is the propagated change of the c -value of cooperatively linked β - and γ -carboxyl groups that gives rise to the observed alteration of K^+_{out}/K^+_{ex} (Ling, 1957, 1958, 1962, 1969).

V. SELECTIVE IONIC PERMEATION

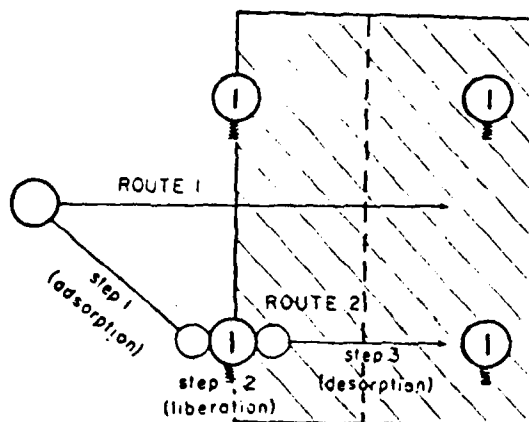
In 1953, I suggested that the basic mechanism of selective K^+ adsorption on the β - and γ -carboxyl groups on the cytoplasmic protein may be extended to β - and γ -carboxyl groups of proteins on the cell surface (Ling, 1953). That the cell surface is endowed with fixed negative charges has long ago been considered by Bethe and Toropoff (1915), Michaelis (1925), Teorell (1935-1936), Meyer and Sievers (1936), Sollner *et al.* (1941), and others. It was new, however, to postulate a high degree of counter-cation association as the basis

of selective K^+ permeability (Ling 1953, 1960, 1962, 1969). In 1965, Ling and Ochsenfeld provided positive evidence that the surface anionic sites may well be β - and γ -carboxyl groups since the rate of K^+ permeation shows an inflection at pH 4.6, the characteristic pK_a of β - and γ -carboxyl groups (Ling and Ochsenfeld, 1965). Entry of K^+ by association of the ion with surface β - and γ -carboxyl groups is followed by liberation of the ion near the fixed anion. Subsequent dissociation and entry into the cell shows "saturation" and "competition." This adsorption-desorption route is one way by which a charged ion of opposite sign to the surface fixed ions may gain entrance into the cell, as is indicated by route 2 in Fig. 5. A second route of entry is via the interstices among the fixed ionic sites, called the saltatory route (route 1).

It has been widely accepted that the cell surface is covered with a continuous lipid layer punctured (Overton, 1899) occasionally with narrow water-filled pores. Yet, in the last 20 years or so, new evidence has been put forth to suggest that this assumption might be true only for certain highly specialized cells, e.g., human erythrocytes (for review, see Ling, 1981b). Among the reasons cited for this view are the following: (1) with few exceptions, e.g., human erythrocytes, most cell membranes do not contain enough lipids to form a continuous bilayer (Ling, 1981b; Jain, 1972, Table 9-2), (2) removal of virtually all lipids did not change the overall thickness or the trilaminar layer spacings of the membranes (Fleischer *et al.*, 1967; Morowitz and Terry, 1969), (3) in the prototypical egg cell (of the frog) the rate of diffusion of labeled water through the cell membrane is equal to the rate of diffusion in the egg cytoplasm (Ling *et al.*, 1967), and (4) at 10^{-7} M the K^+ -ionophore valinomycin reduces the resistance of artificial bilayers of lipids, extracted from erythrocytes, from $10^5 \Omega \text{ cm}^2$ to $2 \times 10^4 \Omega \text{ cm}^2$ in the presence of 30 mM KCl (Andreoli *et al.*, 1967). The membrane resistance of frog muscle cells is $4000 \Omega \text{ cm}^2$ (Katz, 1966, Table 1) of which more than half is due to Cl^- -conductance (Hutter and Padsha, 1959). Thus, the K^+ conductance of frog muscle cells is also in the realm of $10^4 \Omega \text{ cm}^2$. If a substantial part of the muscle membrane barrier is lipid, exposure of frog muscle to 10^{-7} M valinomycin in the presence of 30 mM KCl should cause a substantial increase of inward K^+ flux rate. In fact, none was observed, nor was any change observed in frog ovarian eggs (Ling and Ochsenfeld, unpublished), in squid axon (Stillman *et al.*, 1970), or in the inner membrane of mouse liver mitochondria (Maloff *et al.*, 1978). However, a two-fold increase of K^+ flux rate was observed for human erythrocytes (Ling and Ochsenfeld, unpublished), in agreement with the unusually high lipid content of the red cell membranes (Jain, 1972, Table 9-2).

According to the AI hypothesis, the physiological barrier at the cell surface is multi-

Figure 5. Diagrammatic illustrations of the two routes of ion entry into a fixed-charge system. Shaded area represents a microscopic portion of the surface of a fixed-charge system in which four fixed anions are represented. Route 1 is the saltatory route. Route 2, the adsorption-desorption route, involves a sequence of three steps, adsorption, liberation near the fixed anion, and desorption. This adsorption-desorption route corresponds to the double type, since two ions are involved (the free cation and the fixed anion). (From Ling and Ochsenfeld, 1965, by permission of Biophys. J.)



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layered water polarized by cell-surface matrix proteins with extended polypeptide chains (Ling, 1981a). Membrane lipids serve the role of stabilizing the protein-water system and of simple insulation (Ling, 1977e). This model readily explains why some model semipermeable membranes possess "pores" many times the diameter of solutes to which it is virtually impermeable. Thus, the copper ferrocyanide membrane consists of a network of crystalline particles with an average diameter of 150 Å, and the interstices are expected to be of similar dimensions (see Glasstone, 1946, p. 656). The average pore size of the active layer of cellulose acetate is about 44 Å (Schultz and Asumaa, 1969). Both the copper ferrocyanide and cellulose acetate membrane are semipermeable, highly permeable to water but virtually impermeable to sucrose which has a diameter of only 9.4 Å (Ling, 1973). An additional advantage of the polarized-water model over the conventional lipid layer-rigid pore model is that the cell surface permeability is amenable to the control by cardinal adsorbents (such as Ca^{++} or ATP), which are known to react with cardinal sites (receptor sites) on cell surface proteins.

This model of surface permeation was described by an equation that is a direct extension of Eq. (1), except that, for simplicity, nearest-neighbor interaction was neglected. Under this simplifying condition, the rate of entry of an *i*th monovalent cation into normal muscle cells is described by Eq. (2) (Ling and Ochsenfeld, 1965):

$$V_i = \underbrace{A[p_i]_{\text{ex}}}_{\text{Route 1}} + \underbrace{\frac{V_i^{\text{max}} [p_i]_{\text{ex}} \bar{K}_i}{1 + [p_i]_{\text{ex}} \bar{K}_i + [p_j]_{\text{ex}} \bar{K}_j}}_{\text{Route 2}} \quad (2) \quad \leftarrow \begin{matrix} \text{no} \\ \text{add br.} \end{matrix}$$

where the first and second term on the right-hand side of the equation represent the saltatory route (route 1 of Fig. 5) and the adsorption-desorption route (route 2). $[p_i]_{\text{ex}}$ and $[p_j]_{\text{ex}}$ are the concentration of the *i*th and *j*th cation in the external medium. \bar{K}_i and \bar{K}_j are their respective adsorption constants on the cell surface. *A* and V_i^{max} are both constants. Strong support for this model came from the demonstration that ion entry into living cells as well as model systems of cation-exchange resin sheets, sheeps wool, and even a layer of isolated actomyosin gel are adequately described by this basic equation (Ling, 1960; Ling and Ochsenfeld, 1965, 1970).

VI. MOLECULAR MECHANISMS OF CELLULAR POTENTIALS

The verification of a localized and adsorbed state of K^+ makes a fundamental change of the cellular electrical potential inevitable. Indeed, a new theory of cellular electrical potential based on the early version of the AI hypothesis was already introduced in 1955 (Ling, 1955, 1959, 1960). According to this theory, the cellular resting potential is not a "membrane potential," but is a surface-adsorption potential as was one time suggested in general principle by Baur (see Baur and Kronmann, 1917). However Baur's theory, as a variant of the membrane theory, envisages two such potentials, one on each side of the cell membrane. The AI hypothesis suggests that the entire cell constitutes a proteinaceous fixed-charge system sharing similar attributes due to ion binding and water polarization. Therefore, there is only one surface adsorption potential at the outer cell boundary. It is the density and polarity of the surface β - and γ -carboxyl groups, which also determine the rate of ion permeation, as well as the temperature and the concentration of the external cation that adsorbs onto these anionic sites that determine the magnitude of the resting potential. For resting frog muscle, the simple equation was given by Ling (1959, 1960, 1962, 1967, 1982):

$$\psi = \text{constant} - \frac{RT}{F} \ln \left(\sum_{i=1}^n \bar{K}_i [p_i^+]_{\text{ex}} \right) \quad (3)$$

where $[p_i^+]_{\text{ex}}$ represents the concentration of the i th monovalent cation in the external medium and \bar{K}_i is the adsorption constant of the i th monovalent cation on the surface anionic sites. In a more explicit form, Eq. (3) may be expressed as

$$\psi = \text{constant} - \frac{RT}{F} \ln (\bar{K}_K [K^+]_{\text{ex}} + \bar{K}_{Na} [Na^+]_{\text{ex}}) \quad (4)$$

where \bar{K}_K and \bar{K}_{Na} are the adsorption constants of K^+ and Na^+ , respectively, and should be the same as those in Eq. (2) (\bar{K}_i , \bar{K}_j).

When the surface contains no fixed cation (as I believe is the case in resting muscle), there is no sensitivity to external Cl^- (Hodgkin and Horowicz, 1960), even though muscle has a very high Cl^- permeability (Hutter and Padsha, 1959). Indeed, after eliminating the Cl^- terms from the Hodgkin-Katz equation (Katz, 1966; a procedure I feel not easily defensible), the modified Hodgkin-Katz equation may, for short-term experiment involving little change of internal ion concentration, be reduced to a form entirely analogous to Eq. (3), with permeability constants P_i instead of adsorption constants \bar{K}_i .

$$\psi = \text{constant} - \frac{RT}{F} \ln \left(\sum_{i=1}^n P_i [P_i]_{\text{ex}} \right) \quad (5)$$

Edelmann *et al.* (1971) have determined both the permeability constants and surface-adsorption constants of guinea pig heart with the help of Eq. (2) and concluded that ψ followed Eq. (3) but not Eq. (5) (Edelmann, 1973).

The surface-adsorption model offers an explanation for the data of a large number of reports, including those of Tobias (1950), Falk and Gerard (1954), Kao (1956), Shaw and Simon (1955), Shaw *et al.* (1956), Koketsu and Kimura (1960), and Tasaki and his co-workers (1964, 1965), which indicate that the resting potential does not vary with changes of intracellular K^+ concentration. Other data of Adrian (1956), Baker *et al.* (1961), and Hagiwara *et al.* (1964), show that some dependency of ψ on internal K^+ can sometimes be demonstrated (Ling, 1978b), on the basis of variation of one or more of the following factors: (1) change in surface fixed-ion density (Adrian, 1956), and (2) creation of new amphoteric interface between perfusing or injected fluids and the exposed intracellular protoplasm as well as the degree of ionization of fixed anions (compared to fixed cations).

In 1979, I further revised Eq. (3) to take into account nearest-neighbor interaction among the cell-surface sites (Ling, 1979). The equation for the resting potential now takes the form:

$$\psi = \text{constant}_1 - \frac{RT}{F} \ln \left[\frac{1}{[K^+]_{\text{ex}}} \left(1 + \frac{\xi - 1}{\sqrt{(\xi - 1)^2 + 4\xi \exp(\gamma RT)}} \right) \right] \quad (6)$$

where,

$$\xi = \frac{[K^+]_{\text{ex}}}{[Na^+]_{\text{ex}}} \cdot \bar{K}_{Na} - \kappa \quad (7)$$

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$K_{s2} = \kappa$ and γ have the same significance as in Eq. (1) but refer only to anionic sites on a microscopically thin cell surface. Eq. (6) can account for the gradual decrease of ψ with decreasing external K^+ concentration (and high Na^+ concentration) below that of the normal Ringer's solution (Fig. 6). This theoretical equation is capable of explaining, for example, changes of ψ described by Ruzyllo and Vick (1974) for canine Purkinje cells.

The broader usefulness of Eq. (6) is demonstrated by its ability to explain the experimental data of Maloff *et al.* (1978) who measured the resting potential of the giant mitochondria of cuprizone-fed mouse liver. This potential was virtually indifferent to external K^+ concentration before 10^{-7} M valinomycin was added. Contrary to the expectation based on the membrane theory, in which the expected effect of valinomycin is to increase K^+ permeability of the mitochondrial inner membrane, the absence of change in K^+ conductance was demonstrated (see also Ling, 1981a). On the other hand, these observations can be described by Eq. (6), with the assumption that the affinity for K^+ on the surface anionic sites is increased by valinomycin by a factor of three (For further discussion, see Ling, 1981b).

A more general equation for the resting and action potential would include a diffusion potential term,

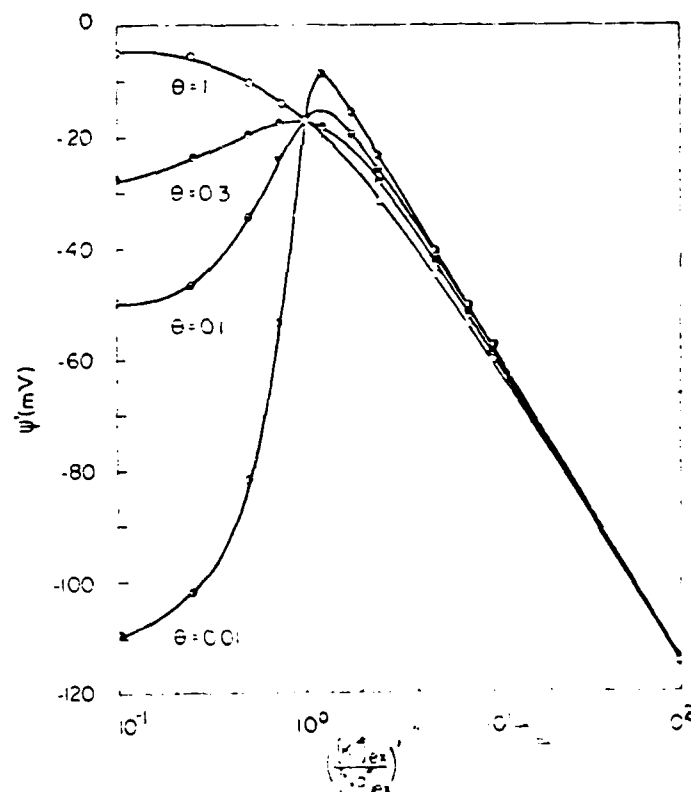


Figure 6. A plot of the resting potential against external K^+ and Na^+ concentration ratio. According to Eq. (6) ordinate represents ψ' which is equal to $\psi - \text{constant}$, abscissa represents $([K^+]_o/[Na^+]_o)^{zK/zNa}$ which is $([K^+]_o/[Na^+]_o) \cdot (K_{s2}^{\gamma}/K_{s1}^{\gamma})$. For experiments carried out in the presence of a constant concentration of Na^+ , e.g., 100 mM, the abscissa is then $[K^+]_o$ ($K_{s2}^{\gamma}/K_{s1}^{\gamma} = 0.1$). Symbols are computed points, prescribed to mark out the separate curves. (From Ling, 1979, by permission of *Physiol. Chem. Phys.*)

$$\psi = \text{constant}_1 - \frac{RT}{F} \ln \left\{ \frac{1}{[K^+]_{\text{ex}}} \left(1 + \frac{\xi - 1}{\sqrt{(\xi - 1)^2 + 4\xi \exp(\gamma RT)}} \right) \right\} \quad (8)$$

$$- \frac{RT}{F} \ln \frac{\gamma_{\text{in}}^{\text{Na}} [\text{Na}^+]_{\text{in}}^{\text{free}} + \gamma_{\text{in}}^{\text{K}} [\text{K}^+]_{\text{in}}^{\text{free}}}{\gamma_{\text{ex}}^{\text{Na}} [\text{Na}^+]_{\text{ex}} + \gamma_{\text{ex}}^{\text{K}} [\text{K}^+]_{\text{ex}}} \quad + 12 \text{ pts } \#$$

where $\gamma_{\text{in}}^{\text{Na}}$, $\gamma_{\text{in}}^{\text{K}}$, $\gamma_{\text{ex}}^{\text{Na}}$, and $\gamma_{\text{ex}}^{\text{K}}$ are the activity coefficients of Na^+ and K^+ in the cell-surface layer water and external solution respectively. $[\text{Na}^+]_{\text{in}}^{\text{free}}$ and $[\text{K}^+]_{\text{in}}^{\text{free}}$ are the free Na^+ and K^+ concentration in the cell-surface water, respectively. The second term on the right-hand

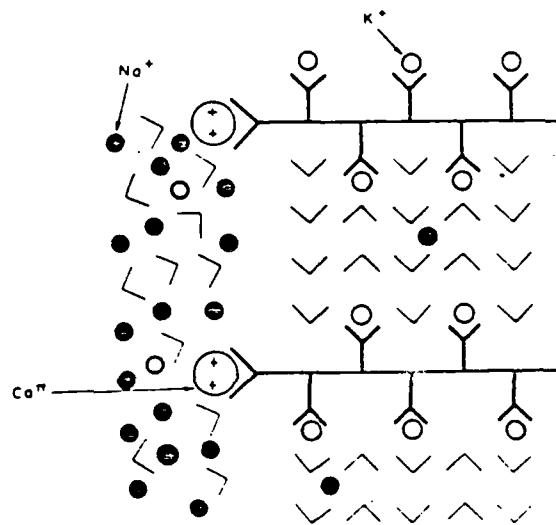
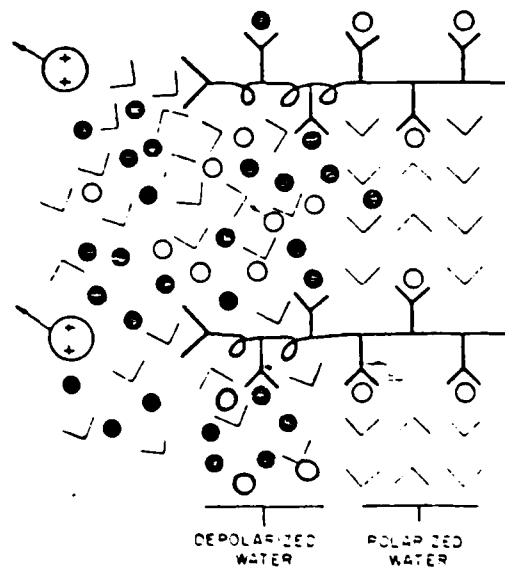


Figure 7. Schematic illustration of the events occurring at the cell surface at rest and during action potential, according to the association-induction hypothesis. The upper figure shows the resting condition, in which K^+ is preferentially adsorbed at the cell surface. Water in the state of polarized multilayers, represented as ordered V's, has low solubility and permeability for both K^+ and Na^+ . Resting condition of preferential K^+ adsorption and polarized water depends on a cardinal adsorbent, Ca^{++} occupying the cardinal site. During activity, Ca^{++} removal causes a time-dependent auto-cooperative change of the electronic conformation of the surface proteins, causing a decrease of K^+/Na^+ preference in response to a c-value increase and the depolarization of water, with a local increase of specific permeability to Na^+ via the adsorption-desorption route and non-specific permeability to Na^+ and other solutes via the depolarized water. (From Ling, 1981c, by permission of Wissenschaftlich Verlagsgesellschaft, Stuttgart).



side of Eq. (8), a diffusion potential term, vanishes when the cell is at rest because under this condition, $\gamma_{in}^{Na}/\gamma_{ex}^{Na} = [Na^+]_{ex}/[Na^+]_{in}$ and $\gamma_{in}^{K}/\gamma_{ex}^{K} = [K^+]_{ex}/[K^+]_{in}$. Now

$$q_{Na} = \frac{\gamma_{ex}^{Na}}{\gamma_{in}^{Na}}, \quad q_K = \frac{\gamma_{ex}^{K}}{\gamma_{in}^{K}} \quad (9)$$

where q_{Na} and q_K are the equilibrium distribution coefficients of Na^+ and K^+ between the surface-cell water and the external solution, respectively. Substituting Eq. (9) into (8), and taking into account of the fact that $\gamma_{ex}^{Na} [Na^+]_{ex} + \gamma_{ex}^{K} [K^+]_{ex}$ is a constant and that $\gamma_{in}^{Na} = \gamma_{in}^{K}$, Eq. (8) simplified to:

$$\psi = \text{constant}_2 - \frac{RT}{F} \ln \left\{ \frac{1}{[K^+]_{ex}} \left(1 + \frac{\xi - 1}{\sqrt{(\xi - 1)^2 + 4\xi \exp(\gamma RT)}} \right) \right\} - \frac{RT}{F} \ln \left\{ \frac{[Na^+]_{in}^{free}}{q_{Na}} + \frac{[K^+]_{in}^{free}}{q_K} \right\} \quad (10)$$

During excitation, K_{Na-K}^{∞} falls due to the c -value increase of the surface anionic sites, q_{Na} (and q_K) concomitantly increases. The inrush of Na^+ brings about not only a cancellation of the resting potential but an overshoot. The adsorption of Na^+ displaces K^+ from adsorption sites at and near the cell surface, causing an increase of free K^+ , i.e., $[K^+]_{in}^{free}$, and hence the delayed outward K^+ current (Fig. 7).

To be noted is the fact that the fixed-surface anionic site would function as a K^+ channel at rest and following activation. But the same sites with a transient c -value change provide an apparent Na^+ channel. Yet in both cases, there is both a noncompetitive ("independent") component through the cell-surface water and a competitive component through the adsorption-desorption route.

The delayed outward current more likely reflects not an opening of a specific "gate" but the diffusion of K^+ after its liberation from adsorption sites at and near the cell surface.

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EXPERIMENTAL CONFIRMATION OF THE POLARIZED MULTILAYER THEORY OF CELL
WATER INCLUDING DATA THAT LEAD TO AN IMPROVED DEFINITION OF COLLOIDS

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SUMMARY

The polarized multilayer theory of cell water, as part of the association-induction hypothesis, and its rapidly gathering supportive experimental evidence were reviewed. It was shown that the new insight offered by this theory reconciles many hitherto unexplained phenomena and that new experimental data also suggest an improved definition of the concept of colloids.

I. GELATIN: THE NAMESAKE OF COLLOIDAL CHEMISTRY

Thomas Graham, who introduced in 1861 the concept as well as the name of colloids, explained in these words, "The plastic elements of the animal body are found in this class. As gelatine appears to be its type, it is proposed to designate substances of the class as colloids..." (*Κολλοῖδ*, the Greek word for glue, is largely gelatine) (Graham, 1861, p. 182). The inventor of dialysis, Graham also stated in the same article (p. 185), "The water of the gelatinous starch is not directly available as a medium for the diffusion of either the sugar or gum, being in a state of true combination, feeble although the union with starch may be..." Beside gelatine, starch and other colloidal materials, Graham also investigated copper ferrocyanide, which is a highly gelatinous reddish-brown precipitate formed when copper sulfate is mixed with K ferrocyanide. It is the study of a membrane of this gelatinous precipitate by M. Traube (1867) and by W. Pfeffer (1877) that led to the founding of the membrane theory. Unfortunately, Traube did not follow the clues Graham revealed but chose to champion the so-called "starch-sieve theory" to explain the impermeability of the

copper ferrocyanide gel membrane to sugars and other solutes. Later x-ray and electron diffraction studies have shown that the interstices of the copper ferrocyanide lattice (100-200 Å) is much larger than that of sucrose (diameter, 9 Å), thus disproving the atomic sieve idea (Fordham and Tyson, 1937; Glasstone, 1946). The semipermeable property of this famous membrane model has remained unexplained until quite recently (see below).

That interaction of biological materials causes changes of property of water was recognized even before Graham's paper of 1861. For example, in 1849, Carl Ludwig, known as the father of modern physiology, demonstrated that dried pig bladder when immersed in a salt solution takes up a much more dilute salt solution than the bathing solution in which the bladder was immersed. This "water of imbibition" or "Schwellungswasser" clearly has different solvent properties from normal water.

In 1934 Holleman, Bungenberg de Jong and Modderman carried out similar study as that described above by Carl Ludwig. However, instead of dried pig's bladder, gelatine was used. Like Ludwig, they also found that water in gelatine gel accommodated, at equilibrium, less Na_2SO_4 than that present in the incubation solution. These authors interpreted their data in terms of "negative adsorption." Similarly when Mc Mahon et al (1940) found lower sucrose concentration in the water taken up by copper ferrocyanide gel than in the surrounding solution they too interpreted the data in terms of "negative adsorption," a term, which I think was not well chosen. Conceptually it is obtuse (see below).

Beside the unusual solvency property, gelatine water system exhibits another distinctive feature: resistance to freezing. In a gel containing 35% water, and 65% gelatine, the water will not freeze even at the temperature of liquid nitrogen (-195.8°C) (Moran, 1926).

In summary, since its very inception, gelatine was seen as having the property representative of colloids including the living matter. These colloidal systems exhibit unusual solvent properties (and have been historically explained in terms of "negative adsorption") and are resistant to freezing.

II. EARLY CONCEPTS OF WATER IN LIVING CELLS

A. Ideas of Pfeffer and Overton

According to the conventional membrane-pump theory of the living cell, all or nearly all water in living cells is free. It is therefore interesting to note that those who first clearly suggested that a substantial portion of the water in living cells is not free but exists as "Schwellungswasser" were in fact the founders

of the membrane theory, W. Pfeffer and one of its great supporters, E. Overton (1902). Overton found that muscle cells when immersed in a solution half of the osmotic strength of a Ringer solution, do not swell to twice their natural size, as would be the case, according to the membrane theory and the van't Hoff equation of osmosis. Overton suggested that a sizable fraction of the water in frog muscle cells is "Schwellungswasser" which was considered to be osmotically inactive. In later quantitative assessment of the amount of this "osmotically inactive" water, the water content of cells is plotted against the reciprocal of the concentration (C) of solutes in the medium. Extrapolating to $C^{-1} = 0$ yields the amount of this fraction of osmotically inactive water. Ling and Negendank (1970) showed that this concept is strictly speaking, incorrect, because it, in fact, says that part of the cell water does not leave the cell even at zero water vapor pressure in the environment. Actual measurement showed that, under this condition, no significant amount of water remains in frog muscle. These findings showed that the kind of cell water behavior contrary to the assumption of the membrane theory as observed by Overton, cannot be reconciled by assuming a portion of it to be osmotically inactive. A better explanation has to be found.

B. Ideas of Gortner

Other proponents of unusual water in living cells included Fisher and Suer (1939) and Gortner (1930). Gortner's work represents a number of physico-chemical studies by him and others of water associated with biological materials. His criteria of "bound water" were based on its nonsolvency for solutes and non-freezing at -20°C . From 1940 on, the bound water idea eventually almost disappeared from the literature in consequence of two types of contradictory experimental findings: (i) urea was found to distribute equally between muscle cell water and the surrounding medium (Hill, 1930); ethylene glycol was found to distribute equally between erythrocyte water and the surrounding medium (MacLeod and Ponder, 1936). These studies led to the conclusion that no bound water exists in living cells. (ii) Blanchard (1940) in his review on "Bound Water" refuted the non-freezing water by pointing out that pure water can be supercooled to temperature lower than -20°C . This and other reasons led Blanchard to reach the conclusion: No bound water exists in living cells.

C. Troshin's Coacervate Theory of Cell Water

A. S. Troshin (1966) from the Soviet Union, was impressed by the findings of Holleman, Bungenberg de Jong and Modderman (1934) mentioned above, confirmed their experiments and suggested that the low levels of sugar, free amino acids, and ions found in living cells have a similar origin: both gelatin and living cell protoplasm represent colloidal "coacervates" systems in which water

has different solubilities for solutes (Bungenberg de Jong, 1949). Regretably this highly talented and productive scientist ceased publishing on this subject in the sixties and he did not further elaborate on how and why coacervates exclude solutes.

III. THE POLARIZED MULTILAYER THEORY OF CELL WATER AS PART OF THE ASSOCIATION-INDUCTION HYPOTHESIS

In 1965 Ling expanded his theory of the living cell, the association-induction (AI) hypothesis to include a more detailed theory of the physical state of the bulk of cell water (Ling, 1965). This theory attempts not only to answer the how and why the bulk of water in a resting cell exists in a different physical state, but also why and how solutes are excluded from water in this state. Before discussing this theory, I shall first briefly sketch its historical background.

A. Historical Background of the Polarized Multilayer Theory of Cell Water

1. Contribution from Physicists. Gases condense on solid surface often in a characteristic manner: a more or less flat plateau is reached at low gas concentrations in the external phase, followed by a steep rise at a higher gas concentration range. Thus this type of adsorption isotherm is \sim -shaped. Physicists De Boer and Zwikker (1929) developed a theory for this phenomenon in terms of multilayer adsorption. This theory was severely criticized by Brunauer, Emmett, and Teller (1938) who pointed out even in the case of very large and hence polarizable atoms like argon or iodine, the solid surface cannot polarize more than one layer of the gaseous molecules. Instead they proposed their own theory (later known as the BET theory) in which additional layers of gases taken up beyond the first adsorbed layer are condensed in the same way as in liquid liquids. However, Brunauer, Emmett, and Teller also specifically pointed out that their criticism was limited to gaseous molecules that have no permanent dipole moments. For gaseous molecules with permanent dipole moments, as in the case of water molecules, polarized multilayer like that suggested by De Boer and Zwikker are quite feasible. Indeed, this case had already been treated by Bradley (1936) who presented a polarized multilayer adsorption isotherm (Eq. 1) in a form quite similar to the De Boer-Zwikker isotherm,

$$\log \left(\frac{p_0}{p} \right) = K_1 K_3^a + K_4, \quad (1)$$

where p is the vapor pressure of the gas under the experimental condition and p_o is p at full saturation. Thus p/p_o is the relative vapor pressure. a is the amount of gas taken up. K_1 , K_3 , and K_4 are constants under a defined condition. Equation 1 can be written in a double log form:

$$\log \left(\log \left(\frac{p_o}{p} \right) - K_4 \right) = a \log K_3 + K_1 \quad (2)$$

2. Contribution from Industrial Physico-chemists. In the textile industry, sorption of water is an important subject of concern. Large uptake by cotton and wool fiber had been for a long time explained as due to capillary condensation; i.e., the bulk of water is held as normal liquid water in pores and narrow channels in the fibers. Benson and Ellis (1948, 1950), in their study of N_2 , O_2 and CH_4 sorption on dry proteins could not find evidence for such pores and channels. In fact they found that the total amount of sorbed gases depends only on the state of subdivision of the proteins and the total surfaces exposed. In sharp contrast, extensive alteration of the physical state of wool, egg albumin, and silk did not alter the amount of water sorbed (Mellon, Korn, and Hoover, 1949). In these cases water uptake depends only on the specific sites available in a protein; the state of subdivisions makes little difference (Benson, Ellis, and Zwanzig, 1950).

From this group of scientists also came the important demonstration that beside polar side chains, the polypeptide chains of proteins offer important sites of hydration, as indicated by the sorption of large amount of water by amorphous poly-glycine ester (Mellon, Korn, and Hoover, 1948) and by polyvinylpyrrolidone

$\left[\begin{array}{c} \text{N} \\ | \\ \text{CH}-\text{CH}_2- \end{array} \right]_n$ which contain no polar side chains (Dole and Faller, 1950). Furthermore, this water uptake by the fibrous proteins and polymers follows Bradley's polarized multilayer adsorption isotherm described by Equation 2.

B. Theoretical Reasons for the Introduction of the Polarized Multilayer Theory of Cell Water

The logical sequence for the polarized multilayer theory of cell water includes the following:

1. In the water of living cells various solutes (e.g., Na^+ , sugars, free amino acids) are found in much lower concentration than in the surrounding medium. There are only three basic types of mechanism for the maintenance of such a difference in concentration between the cell and its aqueous environment. Two of these, absolute membrane impermeability and pumping, have both been ruled out (Ling, 1962, 1983), leaving a difference in the

physico-chemical nature or solubility of the cell internal environment (i.e., water) as the only class of mechanism still tenable.

2. Virtually all cell water must be different, because the concentration of Na^+ and other solutes in the water of many cells may be only 5% of that in the external environment. This sets a limit of normal liquid water in the cells at 5%. Most likely this limit is even lower because it is unlikely that the affected water can exclude Na^+ completely as assumed in this 5% calculation.

3. Cell water comprises from 60 to 85% of the weight of most cells. To convert 95 to 100% of this cell water to something with distinctly different properties, there must be another substance or substances that are at once ubiquitous enough and abundant enough in all cells. Since some cells (e.g., human erythrocytes) contain no DNA or RNA in significant amount, this substance can only be proteins. (This does not rule out a similar role for the nucleic acids and even polysaccharides in some cells to serve a similar role assigned primarily to proteins.)

4. If all the cell proteins are stretched out and uniformly distributed throughout the cell, the space between nearest neighboring chains would be about 20 \AA (Ling, 1962), which is roughly equal to $\frac{20}{2.8} = 7$ water molecules diameters wide. Since not all cells proteins participate, the number of water molecules affected by the involved protein must be greater (but not vastly greater). Thus multilayers of water must be somewhat affected by the cell proteins.

5. One concept introduced of such an influence of proteins on water was the "iceberg" concept (Jacobson, 1955; Klotz, 1958). In this theory protein surfaces offer regular sites that have the geometry of tridymite-like Ice-I structure. This then in some unspecified way induces the formation of more ice layers (at room or body temperature). This theory has been ruled out by the fact that cell water can be supercooled and maintained in that condition but not when the cell water is touched with a seeding ice crystal through a cut end of the cell. Rapid ice formation in cells then follows (Ling and Miller, 1970). This finding shows that no ice could be present in normal cells to begin with (see below).

6. Theoretical considerations discussed in Sect. II, 1. and 2. offer sound basis for the consideration that the bulk of cell water is polarized by cell proteins in the form of multilayers.

C. The Polarized Multilayer Theory of Cell Water and the Theory for Solute Exclusion from Water in This State

The polarized multilayer theory of cell water first proposed

in 1965 as part of the AI Hypothesis (Ling, 1965) was amplified in 1973 and 1975 (Ling, 1973, Ling and Sobel, 1975), includes the following postulations.

1. In the intracellular space of all living cells not occupied by other matter, a network of regularly and finely dispersed proteins, referred to as "matrix proteins" exists.

2. The matrix proteins exist in an extended conformation with their backbone NHCO groups directly exposed to the bulk-phase water. The NH and CO group, being positively charged (P) and negatively charged (N) respectively constitute a NP-NP-NP system.

3. Each of these CO groups and its neighboring NH group orient rows of water molecules with opposite orientations. As a result there is not only radical polarization of the water molecules along each row but also lateral interaction between water molecules in immediately neighboring rows. The entire assembly thus assumes 3-dimensional cooperative characteristics.

4. As a result of the multilayer polarization, the water molecules have reduced rotational as well as translational motional freedom.

5. The electrical polarization of the assembly of water molecules and the motional restriction thus created provides two basic mechanisms for the exclusion of large solute molecules from this polarized water: enthalpic and entropic. In both mechanisms, small solutes and solutes that can fit into the water lattice may not be excluded at all or may even be preferentially taken up. The degree of exclusion as measured by the equilibrium distribution coefficient (q-value) increases with increasing molecular sizes and complexity (the size rule).

6. The degree of motional restriction considered is very modest (Ling, 1979). Thus to produce a q-value of 0.1 for a solute (which is quite low for q-values), the required motional restriction, represents no more than a factor of 10 in the "partition function" ratios. This type of water structure is dynamic, the long-range order can be revealed only after many repeated photographic exposures are taken.

IV. EXPERIMENTAL TESTING OF SEVERAL OF THE PREDICTIONS OF THE THEORY

A. Solvency

A corollary of polarized multilayer theory of cell water is that when a protein exists in an α -helical or other intramacro-

molecularly H-bonded structure, the bulk phase water will not be influenced. As a result, even large solutes like hydrated Na^+ , sugar, etc., will either not be excluded at all or minimally excluded. This theoretical corollary was confirmed by Ling, Ochsenfeld, Walton, and Bersinger (1980).

However, when either due to specific non-helical molecular structure, or exposure to denaturants like urea or guanidine HCl, the backbone NHCO groups of a protein are exposed to the bulk phase solvent, solute exclusion then is observed.

From these studies, the conclusion was also reached that the reason water associated with gelatin excluded Na^+ and other solutes, normally excluded from living cells, is due to gelatin's existence in an extended conformation, as a result of the possession of the repeated triads of non-helical forming amino acid residues: glycine, proline, and hydroxyproline. In full agreement, a number of synthetic polymers including polyvinylmethyl ether

(PVME) $(-\text{CH}-\overset{\text{H}}{\underset{\text{O}-\text{CH}_3}{\text{C}}})_n$; polyvinylpyrrolidone (PVP); poly(ethylene oxide) (PEO) $(-\text{CH}_2-\text{O}-\text{CH}_2)_n$; and methylcellulose (Ling, Walton, and Bersinger, 1980) which do not contain an H-donating group like NH in the polypeptide chain also alter the solvency of the bulk-phase water. These findings show that all that is needed to produce the solvency effect on the bulk-phase water is the presence of unshielded oxygen atoms with its lone pair electrons along the chain at distance roughly equal to that of two water diameters.

In agreement with this theory it was found that water associated with PVME, does not exclude methanol but excludes larger hydroxylic compounds in rough proportion to the sizes and complexities in full agreement with the size rule long ago reported for living cells (Ling, Miller, and Ochsenfeld, 1973).

B. Degree of Motional Restriction of Polarized Water in Model Systems

The NMR relaxation time, T_1 and T_2 of the proton of water associated with PVP, PVME, and PEO were studied (Ling and Murphy, 1982). The T_1/T_2 ratios are very close to that of pure water (i.e., near unity). This equality of T_1 and T_2 and the actual values of T_1 and T_2 at the magnetic field used, permits a rough estimate of the rotational correlation time (τ_c) which turns out to be no more than 1 order of magnitude longer than normal liquid water and thus vastly shorter than that of ice.

C. Swelling and Shrinkage of Polymer-Water Systems and of Living Cells Without Intact Membrane

Dialysis sacs filled with a 30% solution of the neutral polymer (PEO) maintain a swollen, unchanged, or shrunken volume depending on the concentrations of Na-citrate in the external solution even though the dialysis sac is fully permeable to Na-citrate (Ling, 1980). This confirms the theoretical expectation that it is primarily the multilayer polarization and hence lowered activity of the polymer-dominated water in conjunction with the low equilibrium distribution coefficient or q -value of Na-citrate in the polymer-oriented water that determine the volume of the sacs. A similar mechanism was suggested in the AI Hypothesis for the maintenance of the size of living cells, which have been shown to swell or shrink regardless of the presence or absence of an intact cell membrane (Ling and Walton, 1976).

D. Freezing Properties

Ling and Zhang in work yet to be published showed that the non-freezing water in gelatin-gel is also found in water associated with PEO and other polymers. They came to the conclusion non-freezing is yet another trait of the water polarized in multilayers. These data also clearly showed that the polymer-oriented water is definitely not ice as postulated in the iceberg theories.

E. Adherence to the Bradley Adsorption Isotherm

Ling and Negendank (1970) showed that 95% of the water in isolated frog muscle cells follow Bradley's adsorption isotherm (Equations 1 and 2). In the last six years, three different laboratories across the world using four modern methods, showed that the bulk of the major intracellular cation K^+ is adsorbed (Edelmann, 1977, 1980-81, 1981; Ling, 1966; Trombitas and Tigyi-Sebes, 1979). Yet the cells are in osmotic equilibrium with a Ringer solution which has an osmotic strength equal to that of a 0.118 M NaCl solution in which both Na^+ and Cl^- are free. This osmotic equilibrium with a Ringer solution is maintained whether the muscle is in direct contact with the Ringer solution or separated from the Ringer solution by an air space as was the case in the experiments described by Ling and Negendank (1970). In terms of the AI Hypothesis, these observations indicate that the osmotic balance is maintained primarily by the matrix proteins. Although not yet clearly identified, it is believed that actin, tubulin, and other cytoskeletal proteins may play major roles. However, it is also believed that they exist in a finer state of dispersion than seen in EM pictures. In other words we expect that the matrix proteins have properties illustrated, though less intensely, by

with an average diameter of 45 \AA (Ling, 1973) which is 5 times wider than the diameter of sucrose, to which the membrane is virtually impermeable, clearly the situation closely resembles the case of copper-ferrocyanide gel membrane. In both, a low q -value of sucrose for the water polarized by either the cellulose acetate, or by the copper-ferrocyanide, in addition to greatly reduced diffusion coefficient (D) for sucrose in this polarized water accounts for its low permeability ($P = qD$). Of course, q -value as well as D -value decreases as the size and complexity of the hydraulic compound decreases. Therefore the permeability of these hydroxylic compounds are progressively higher for the compounds with smaller size. Water, having a q -value of 1.0 and apparently a correspondingly high D , is the most permeable through both models and the living cell membrane.

B. An Amplification of the Term, Negative Adsorption

With multilayer adsorption of water established, the term that a solute is "negatively adsorbed," can be better understood. It signifies that like Na^+ , glucose and free amino acids, a negatively adsorbed solute is that which is excluded from the polarized multilayers. However, the term remains a poor one, first, in the sense the solute excluded does not have direct contact with the polarizing surface and thus is not at all AD-sorbed. Second, negative adsorption suggests that the surface sites actually repel it, which they don't.

C. Overton's Schwellungswasser in Living Cells

Overton was forced to postulate that a portion of the cell water to be different and in later investigator's lingo, osmotically inactive. I already pointed out that this is not a sound idea and is refuted by actual measurements. However, the basic trouble originates from the assumption that the bulk of cell water is normal liquid water and in which free solutes like K^+ determine its osmotic strength according to van't Hoff's law. Since these assumptions have proven invalid and since the volume change is primarily a matter of multilayer adsorption of water (Eq. 1, see also Ling and Peterson, 1977) clearly no postulate of part of the water being osmotically active can explain the full behavior pattern.

D. Urea and Ethylene Glycol Are Not the Proper Probe for Water Existing in the State of Polarized Multilayers

The "bound water," as used by earlier investigators, was given attributes that were incorrect. Among those was the attribute of categorical non-solvency - i.e., non-solvency for any solutes. It was by siezing this incorrect assumption, that the

bound water concept of Gortner and others was "disproven" by Hill, and MacLeod and Ponder mentioned earlier. According to the polarized multilayer theory, small molecules and molecules that can fit into the water lattice are not excluded. These non-excluded solutes experimentally demonstrated include urea and ethylene glycol (Ling, Walton, and Bersinger, 1980), which were respectively that used by Hill (1930) and by MacLeod and Ponder providing the major evidence against the bound water ideas of the 1930's.

VI. HOW CAN WE DELIBERATELY FORGET THE MAJOR ADVANCEMENT MADE IN UNDERSTANDING WATER STRUCTURE ON THE BASIS OF ITS TETRAHEDRAL H-BONDED STRUCTURE?

Bernal and Fowler (1933) proposed the first modern structural theory of water. That liquid water retains short-range order is now well established. The tetrahedral structure of water molecule underlies a natural tendency to form 4 pairs of H-bonds with neighboring water molecules like in Ice I. Although no consensus of liquid water structure has been reached yet, this basic element of tetrahedral structure is universally accepted (Eisenberg and Kauzmann, 1969). The question may be raised, "Why should we now propose a modified liquid water structure, which seems to ignore these important detailed knowledge?"

At the time when French impressionists started to make their unorthodox artistic renditions of nature, they might well have been asked a similar question, "Why do you ignore the knowledge we already have gained in detail painting, in preference for the crude pictures you seem to prefer?"

My answer to the criticism of the choice of the dipole-dipole model over the more detailed H-bonded model may be similar to one that could have been offered by the French impressionists: "In order to see the whole forest, details must be deliberately ignored."

Equally important, the dipole-dipole interaction is preferred over the tetrahedrally-H-bonded water molecule interaction because at this moment we can predict certain properties of water on the basis of the dipole model which we cannot when based on the complicated tetrahedral structure. Thus dipole-dipole interaction may predict in one way of orientation, an attraction between two water molecules, and in another orientation, there can be repulsion. The present-day model of the H-bonded structure predicts attraction all right but would be strained to describe less favorable interaction than no interaction. In short, the dipole-dipole interaction model is, at this time at least, correct, highly useful, and informative in the same sense, an impressionist picture of a

landscape is more representative than a picture in which every leaf of a tree has been painted.

VII. A REVISED DEFINITION OF COLLOIDS

Graham defined colloids as materials having the properties of gelatine. In years following, the development of colloidal chemistry emphasized the large molecular size of colloidal materials. Yet we have now amply shown that gelatin does indeed stand apart from native proteins with similar molecular weights and that gelatin owes this unusual property not to large molecular weights (witness copper ferrocyanide is a colloid, like gelatin but having a very small molecular weight) but to its ability to orient multilayers of water on its extended polypeptide chains. I would therefore like to suggest the colloids as envisaged by its founder Thomas Graham are distinguished by their ability to achieve multi-layer interaction with the bulk phase solvent, water.

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STUDIES ON THE PHYSICAL STATE OF WATER IN LIVING CELLS AND MODEL SYSTEMS.

V. FURTHER STUDIES OF THE WARMING EXOTHERMIC REACTION OF FROZEN AQUEOUS SOLUTION OF POLYVINYLPIRROLIDONE, POLY(ETHYLENE OXIDE), AND UREA-DENATURED PROTEINS

by

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INTRODUCTION

In the preceding paper, Ling and Zhang (1983) have shown that gelatin, urea-denatured globular proteins, polyvinylpyrrolidone (PVP), poly(ethylene oxide) (PEO), and polyvinylmethylether (PVME), all of which produce solvency reduction of water (Ling et al, 1980a, b; Ling and Ochsenfeld, 1983), also cause freezing-point and thawing-point depression of the water, a progressive widening of the freezing peak and a warming exothermic peak during thawing. In contrast, globular proteins and globular proteins denatured by sodium dodecyl sulfate (SDS), none of which produce solvency reduction of water, have only minimal or no effects on the freezing and thawing temperatures of bulk phase water, their freezing peak widths, nor do they produce warming exothermic peaks.

Warming exothermic peaks to be referred to as WEX, were reported by Luyet, Rasmussen, and Kroemer in 1966 in aqueous solutions of glycerol and ethylene glycol (Luyet et al, 1966) and later by Luyet and Rasmussen (1967) in aqueous solution of PVP. Luyet and coworkers referred to the underlying mechanism as "devitrification," a change from amorphous ice to crystalline ice. Later Cooks et al (1975) reported a similar observation during the thawing of "frozen" ternary systems of H_2O -NaCl-glycerol and of H_2O -NaCl-dimethylsulfoxide (DMSO). All of these studies were at least partly motivated by an interest in the mechanism how glycerol, ethylene glycol, PVP, and DMSO exercise their well-known cryoprotective effects.

In the present paper we shall present results of a more detailed study of the WEX phenomenon in protein-water and polymer-water systems, using the technique of differential scanning calorimetry.

MATERIALS AND METHODS

The materials used in this investigation and their sources are essentially the same as described in the preceding paper (Ling and Zhang, 1983). Polyvinylpyrrolidone (PVP-360) was from Sigma Chemical Co., polyvinylmethylether (PVME) from GAF Corp., poly(ethylene glycol) (Polyox WSR-205, M.W. 600,000, Polyox WSR-N750, M.W. 300,000) from Union Carbide; urea from J. T. Baker; gelatin from Eastman; hemoglobin (Lot 12F-9322), bovine serum albumin (Lot 70F-9380), protamine sulfate (Lot 100F-9390), and pepsin (Lot 60F-8057) were all from Sigma Chemical Co.

The calorimetric measurements were made on a Perkin-Elmer DSC-2 differential scanning calorimeter. Temperature as low as 223° K was obtained by immersing the cooling block in a mixture of dry ice and 95% ethanol. As a rule, samples were cooled or heated at the cooling or heating rate of 10° /min. unless otherwise stated. The sensitivity of the calorimeter was 10 mc/sec. Water and indium were used for calibrating the temperature.

RESULTS

Figure 1 shows the thawing DSC thermogram of polyvinylpyrrolidone (PVP) solution in concentrations ranging from 9% to 60%. The 50% PVP thermogram is virtually the same as that reported for 50% PVP by Luyet and Rasmussen (1967). The more complete data presented here covering a variety of PVP concentrations show variability of the thawing behavior with even minor changes of the polymer concentration. Note the absence of the warming exothermic peak (WEX) at 43% PVP concentration in contrast to the pronounced WEX in a solution only 6% stronger. From 49% to 51% there was another marked change in the size of the WEX and temperature at which it occurs. At 60%,

neither WEX, nor observable endothermic thawing peak could be seen. Note also that in concentrations at and below 43%, and before the onset of WEX, there was small endothermic peaks which moved to lower temperatures as the concentration of PVP increased.

Figures 2 and 3 are thawing DSC thermograms of two types of poly (ethylene oxide) (PEO). Only in solutions of Polyox WSR-205 was the WEX observed; in solutions of Polyox WSR-N750, no WEX was visible even at a PEO concentration of 69.4%. In the thawing scans of 9 or 10% solution of PEO there were secondary endothermic peaks at temperature below that at which the main thawing peaks occurred. These secondary peaks resemble those seen in 23% and 33% PVP though they were prominent and grew more so until at still higher concentration, they merged with the main endothermic peak as the main endothermic peak moved progressively to lower temperatures.

The moderate decrease of the thawing temperature in solutions of increasing urea concentration (Fig. 4) contrasts with much larger changes of their freezing temperatures (Ling and Zhang, 1983). Figures 5, 6, and 7 are the thawing thermograms of urea-treated hemoglobin (28.5%), bovine serum albumin (BSA) (28.5%), and protamine sulfate (28.5%) respectively. Exposure to 3 M urea produced a lowering of the melting temperature and a broadening of the peak for all these proteins. A WEX appeared in 28.9% hemoglobin treated with 5 M urea; the WEX became sharper at higher urea concentrations (Fig. 5). The appearance of WEX began at 7 M for BSA (Fig. 6) and at 9 M for protamine sulfate (Fig. 7). Thus exposure to 9 M urea, converted the globular proteins with sharp melting peaks to the complex pattern of exothermic peaks followed by the endothermic melting peak, very similar to that seen in 49% PVP and in 40% PVME described in the preceding paper (Ling and Zhang, 1983).

Figure 8 shows the effect of pepsin concentration on the WEX phenomenon. An aqueous solution of 28.5% pepsin and 9 M urea did not exhibit a WEX; the thawing peak was quite similar to that of 28.5% pepsin except its shift to a lower temperature presumably on account of activity of urea. It should be mentioned that resistance of pepsin to urea denaturation has long been known (Lineweaver and Schwimmer, 1941). However a 9 M urea solution containing 50% pepsin showed a fully developed WEX. Since 28.5% hemoglobin, 28.5% bovine serum albumin as well as 28.5% protamine sulfate all exhibited a WEX in the presence of 9 M urea, clearly the concentration of proteins needed in the presence of 9 M urea to create a WEX varies with the nature of the proteins.

The Cooling Rates and the Appearance of WEX

Luyet and Rasmussen (1967) believed that the WEX phenomenon represents a devitrification phenomenon. In this view, WEX can only occur if the water in an aqueous solution is caught in an amorphous state by very rapid cooling and becomes vitrified ice. We tested their hypothesis by altering the cooling rate of 50% PVME between 320 degree per minute to 256 times slower (i.e. $1.25^{\circ}/\text{min}$). The same WEX was observed in all cases as shown in Fig. 9, in contradiction to the expectation based on the devitrification theory.

Effect of the Duration of Prolonged Exposure to 223°K on the WEX

Although WEX does not depend on the rate of cooling in the range studied, it did vary with the time that the "frozen" solution is kept at 223 K (-50°C).

Thus as shown in Fig. 9, 1 min. after cooling to 223 K, 49% PVP-360 showed a pronounced WEX, which, however, became progressively less prominent following further stay at this temperature until after one hour the WEX disappeared altogether. A small secondary endothermic peak can be then discerned which may well be the same kind of secondary endothermic peaks seen in Fig. 1 in PVP-360 at concentration up to 43%.

In the case of 50% PVME (Fig. 11) it took about 2 hours at 223 K for the WEX to disappear. After that a double headed endothermic peak remained. Comparing with Fig. 6 of the preceding paper, the high-temperature peak appeared to be the main melting peak moved to lower temperature and the low temperature peak may be the same secondary endothermic peak, now sitting on the shoulder of the main peak moved to a lower temperature.

In contrast to the two polymers, urea denatured hemoglobin was the slowest to lose its WEX (Fig. 12). The rates of disappearance of WEX, expressed as a ratio of the area of WEX over that of the main endothermic peak is shown in Fig. 13. One recalls that urea by itself does not produce a WEX (Fig. 4).

The Melting-point Depression of Globular Native Proteins, Gelatin, and the Three Polymers, PVP, PEO, and PVME

In the preceding paper, Ling and Zhang have shown how, as a general rule, bulk phase water in solutions of native globular proteins freeze at more or less the same temperature while PVME, gelatin, and urea-denatured proteins do significantly depress the freezing point of the bulk phase water. The freezing point is prone to be affected by a variety of extraneous factors, e.g., sample size, shape, and size of containing vessels, etc. However, these factors that bring about non-equilibrium or supercooling, does not apply to thawing since it does not involve the movements of water molecules from a

more random liquid state to a more ordered frozen state, but rather it involves the opposite change in which macroscopic barriers and other factors do not affect the process.

DISCUSSION

Vitreous Ice vs. Polarized-multilayer Water

Luyet, Rasmussen and their coworkers (Luyet et al, 1966; Luyet and Rasmussen, 1967) first reported what we now refer to as the WEX phenomenon in aqueous solution of glycerol, ethylene glycol, and PVP. They explained the phenomenon as due to "devitrification," contending that it was a prior rapid cooling that created the vitreous ice from normal liquid water in the sample and that it was the transformation of this vitreous ice into normal Ice I during rewarming that gives rise to the heat-releasing WEX.

There are several reasons that argue against this interpretation:

(1) Supercooling cannot create vitreous ice: Kamb (1972, p. 11) wrote: "The vitreous form of ice has been made by condensation of water vapor below -150°C , but it is impossible, as far as we know, to convert liquid water to a vitreous form by rapid cooling."

(2) The coexistence of water with large quantity of macromolecules (e.g., 50% globular protein solutions) does not necessarily impede normal ice formation (Ling and Zhang, 1983).

(3) WEX does not depend on rapid cooling: A 250 fold slow-down of cooling rate did not alter WEX of PVME solution (Fig. 9) and left little doubt that WEX did not originate from very rapid cooling.

(4) Water in chilled PVME solution is not in the state of ice: Vitreous ice like all other ices by definition is a solid and rigid. Yet a drop of 50% PVME remains as a viscous liquid and yields readily to manipulations with the glass rods when held between a pair of glass rods and immersed

in a mixture of ethanol and dry ice kept at about -70°C .

An Alternative Interpretation of the WEX

Luyet, Rasmussen and their coworkers believed that water in the aqueous solutions of PVP, gelatin, glycerol, etc., is normal liquid water. However, normal liquid water is not known to have been supercooled to beyond -40°C (Dorsey, 1940; Hallet, 1965). That is to say, in cooling solutions of PVP and gelatin to that of -70°C (i.e., temperature produced by the dry-ice alcohol mixture), Ice I should have been formed instantly, contrary to the facts. This then led Luyet and coworkers to the postulation that rapid cooling turned the water in these systems into vitreous ice. Clearly at that time they shared the common belief, now disproven (Ling and Zhang, 1983), that the presence of macromolecules invariably slows down the rate of ice-formation.

The purpose in postulating the existence of vitreous ice is to produce at temperature far below the freezing point of normal water, a condition which keeps the water molecules from such rotating and diffusing motions necessary to transform them into Ice I. The explanation we would like to offer is that in the presence of gelatin, PVP, PVME, etc. of suitable concentrations, the bulk of water does not exist as normal liquid water. Rather it exists in the state of polarized multilayers and cooling to -50°C of water in this state does not promptly turn this water into Ice I (see below) because there is a substantial energy barrier between these two states. To overcome this energy barrier, one can increase the temperature and hence the average kinetic energy kT of the water molecules as one does during warming. This is why during a WEX heat is given off during warming in apparent contradiction

to the Le Chatelier principle. Another way to achieve the transformation from the chilled multilayer state to Ice I state is by lengthening of the time of exposure to -50°C so that eventually all the water molecules come across the energy barrier, albeit slowly. As mentioned above, this expectation too, has been confirmed (Figs. 10, 11). An implicit assumption of the present explanation is that the state of Ice I, though not immediately reached, nevertheless does represent a lower energy and hence more stable state. However, in very concentrated solution of water-polarizing polymers (e.g., 65% PVME, see Fig. 6 of preceding paper), this may not be the case. Here, neither freezing peak, nor WEX, nor thawing peak was observed. Apparently water is energetically more favorable in the polarized multilayer state in the presence of 65% PVME than Ice I state at the low temperatures studied.

The Involvement of Polymer Chain Translation and Rotation in the WEX Phenomenon

60% PVP (Fig. 1), and 65% PVME (Figs. 2 and 4 of Ling and Zhang, 1983) ← do not form ice under all the conditions studied. Nor do they freeze, demonstrate WEX, or thaw. Yet 53% PVP does exhibit a WEX, though a weak one. The only difference between a 53% PVP and a 60% PVP is 7% additional PVP and 7% less water. Clearly the presence of PVP has greatly reduced the tendency to form ice either during cooling or warming. Since the physical state of water in a 53% PVP and a 60% PVP can hardly be fundamentally different, it is more likely that the difference lies in the greater degree of entanglement or other stabilizing influences the few additional percentage of PVP adds to the already dense PVP-chain assembly. If this explanation is correct, it would suggest that due to the stronger interaction between the water multilayers and the polymer chains, for water in the chilled polarized

multilayer state to transform to Ice I, it is not just water molecules that have to undergo the proper motions but that the polymers must also move in unison. When this movement is sufficiently hindered, as it seems to be in the 60% PVP or 65% PVME, the water will remain in the polarized multilayer state during "freezing" and will not change to Ice I.

The concept of a mandatory requirement of coordinated movements of the polarizing polymers with water in the WEX phenomenon is significant in that this idea can help to resolve the unusual behaviors of PEO (especially Polyox N750).

PEO shares the same solvency reduction effect with PVP, PVME, and gelatin. Indeed, PEO is one of the most effective among these polymers and proteins. It differs from PVP, PVME, and gelatin in several characteristics:

(1) Unlike PVP, PVME, and gelatin, which at high concentrations do not freeze at all, PEO does freeze at a concentration as high as 69.4% (Ling and Zhang, 1983, Fig. 3). The explanation of this apparent anomaly is that in contrast to all the other polymers, gelatin, and urea-denatured proteins, all of which possess side chains that can impede rotational motional freedom, PEO, a polymer containing only the repeating units of $\text{-(CH}_2\text{-O-CH}_2\text{)-}$ has no side chains. It is therefore logical to expect the PEO chains to rotate or otherwise move more readily than polymers with side chains to follow the movements of water molecules toward the Ice I state.

(2) Polyox WSR-N750 showed no WEX. Polyox WSR-205 (MW 600,000) which is on the average, twice as long as Polyox WSR-N750 (MW 300,000) demonstrated a WEX, whereas none could be seen in the case of Polyox WSR-N750 at all. Clearly with the easy rotation and other motions of Polyox WSR-N750, all the polarized water has become transformed to Ice I during the freezing process and in the subsequent brief

period of time of incubation at 223° K. The longer Polyox WSR-205 molecules do impose some restriction on freezing due to the greater energy barrier for the chain movement than Polyox WSR-N750. As a result Polyox WSR-N750 even at as high a concentration as 69.4% could rapidly form ice during the freezing at 10° /min whereas a 60% Polyox WSR-205 would require a higher kT provided by the warming to execute the same required motional adjustments for ice formation.

SUMMARY

The warming exothermic peaks (WEX) first described by Luyet and co-workers in solutions of ethylene glycol, glycerol, and polyvinylpyrrolidone (PVP) was interpreted on the basis of a devitrification phenomena, i.e., rapidly cooling converts normal water to amorphous ice, which devitrifies on warming to the state of normal Ice I. Our present studies of the WEX phenomenon in aqueous solution of urea-denatured proteins, PVP, and two types of poly(ethylene oxide) led us to a different conclusion: WEX is the result of a transformation of chilled water in the state of polarized multilayers to Ice I. Among the evidences cited is the fact that WEX of the polymer-water system is indifferent to the rate of cooling between 320° /min. to 1.25° /min. and that polymer-water systems cooled to -70° C remain a viscous liquid and not (solid) vitreous ice.

The transformation from water dominated by PVP, PVME, and urea-denatured proteins to Ice I can occur rapidly during warming or more slowly when the chilled polymer-water systems are kept at 223° K.

Evidence was also presented showing that to affect this change the polymers themselves must also undergo movements which tend to be retarded most by the presence of bulky side chains as in urea-denatured proteins but is retarded least in polymers with no side chains (e.g., Polyox WSR-N750).

ACKNOWLEDGMENT

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LEGENDS

- Figure 1 - DSC thermograms of various concentration of PVP-360 solutions during thawing.
- Figure 2 - DSC thermograms of various concentrations of PEO (Polyox WSR-205) (MW 600,000) solutions during thawing.
- Figure 3 - DSC thermograms of various concentrations of PEO (Polyox WSR-N750) (MW 300,000) solutions during thawing.
- Figure 4 - The thawing thermograms of solutions of urea of varying concentrations.
- Figure 5 - DSC thermograms of urea-denatured hemoglobin (28.5%). Concentration of urea indicated in molarity.
- Figure 6 - DSC thermograms of urea-denatured BSA. The concentration of BSA solution is 28.5%. Numbers represent the molar concentrations of urea solutions.
- Figure 7 - DSC thawing thermograms of 28.5% aqueous protamine sulfate solutions in the presence of different concentrations of urea.
- Figure 8 - DSC thawing thermograms of urea-denatured pepsin. The concentrations of pepsin are given in percentage and those of urea in molarity.
- Figure 9 - DSC thermograms of thawing 50% PVME after cooling down to 223° K at various cooling rates as indicated.

Figure 10 - WEX decay. 49% PVP-360 solutions were held at 223° K for varying lengths of time as indicated before warming began.

Figure 11 - WEX decay. 50% PVME solutions were held at 223° K for different lengths of time as indicated before warming began.

Figure 12 - WEX decay. Hemoglobin in 9 M urea was held at 223° K for varying lengths of time before warming began. Concentration of hemoglobin was 28.5%.

Figure 13 - Decay of WEX. The area of the integrated area of the warming exothermic peak (S_{WEX}) expressed as a ratio to the integrated area of melting peak (W_{WEX}) for PVP-360 (O), PVME (Δ), and urea-denatured hemoglobin (X).

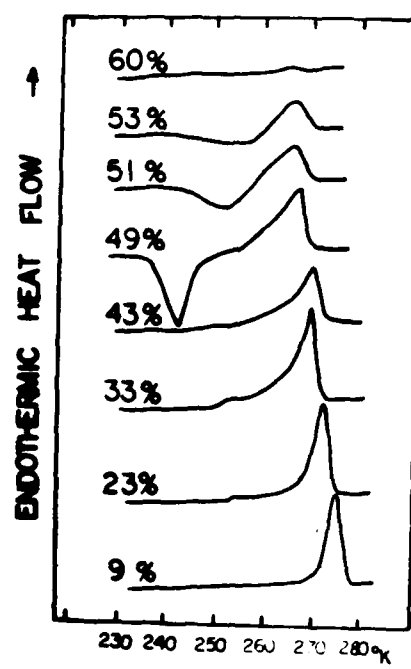


FIGURE 1

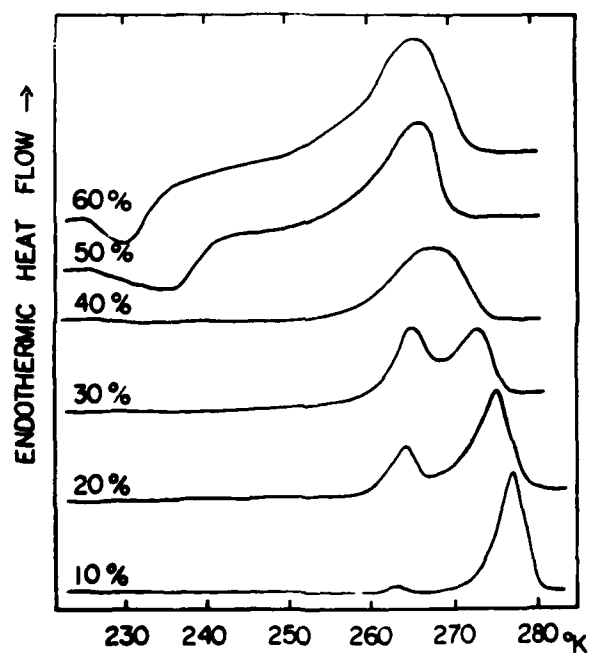


FIGURE 2

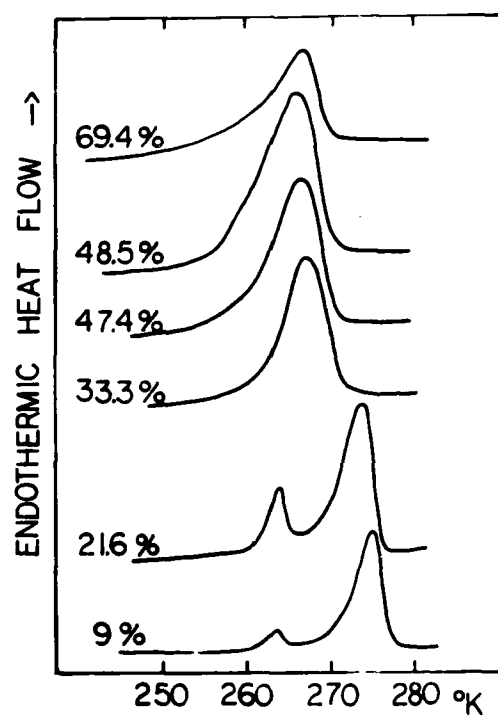


FIGURE 3

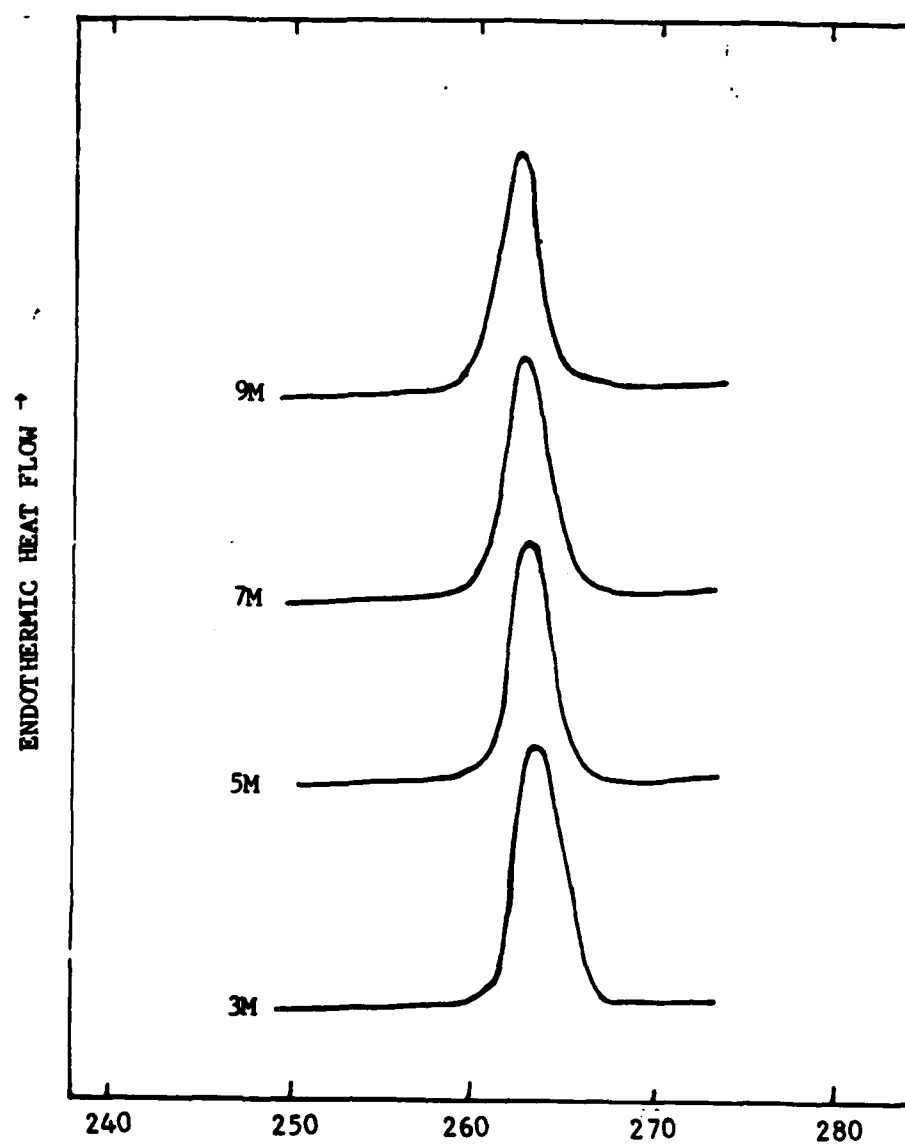


FIGURE 4

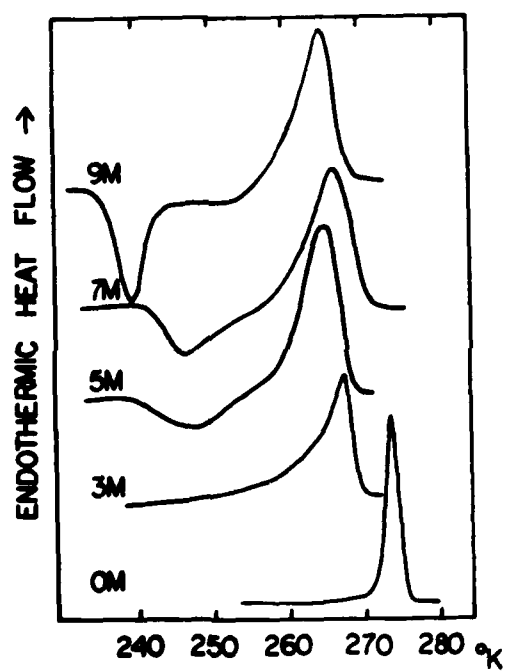


FIGURE 5

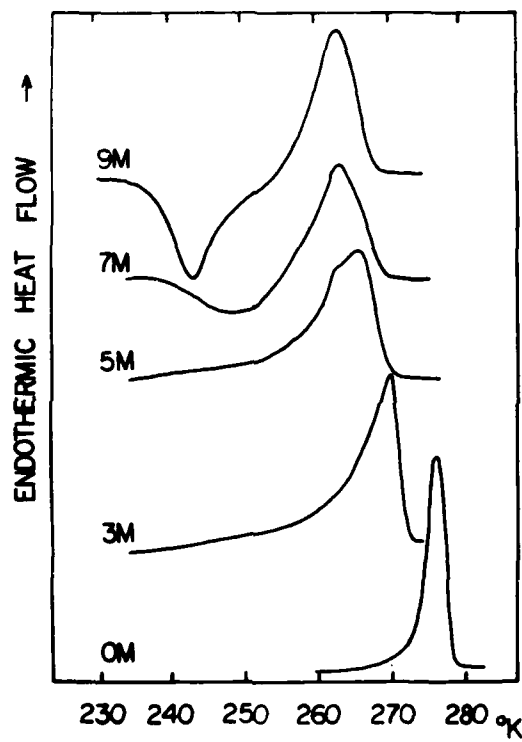


FIGURE 6

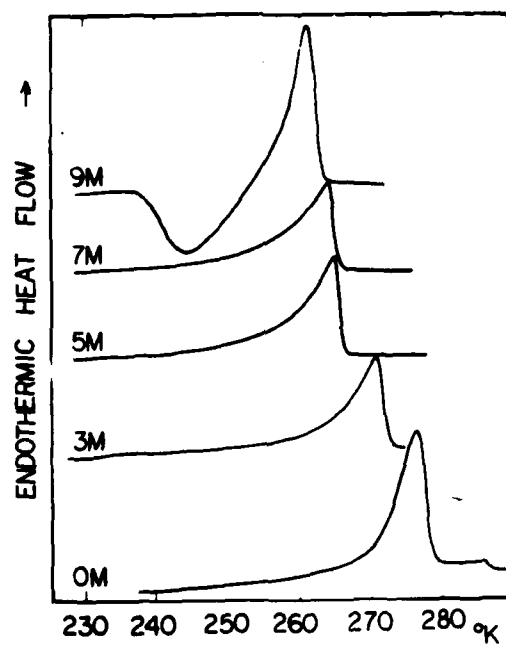


FIGURE 7

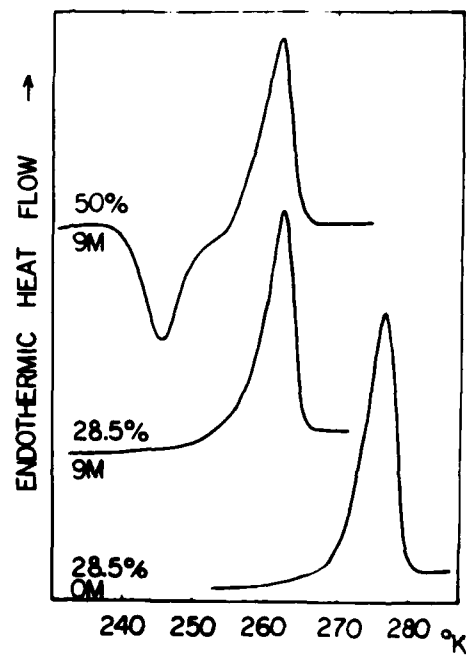


FIGURE 8

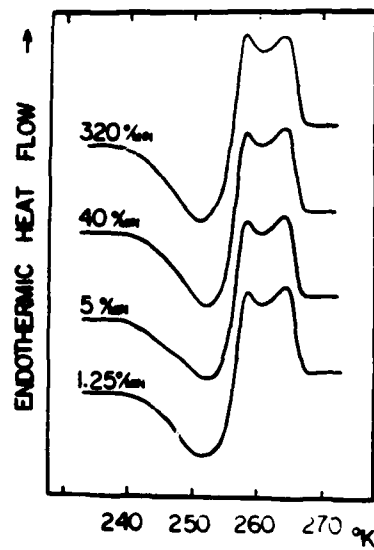


FIGURE 9

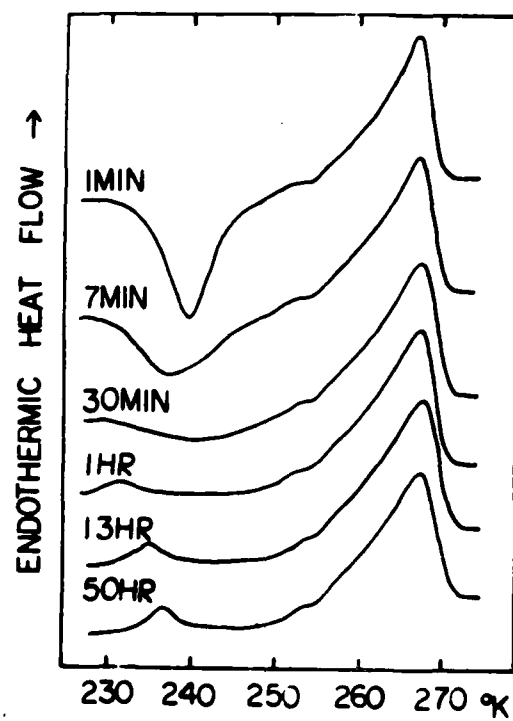


FIGURE 10

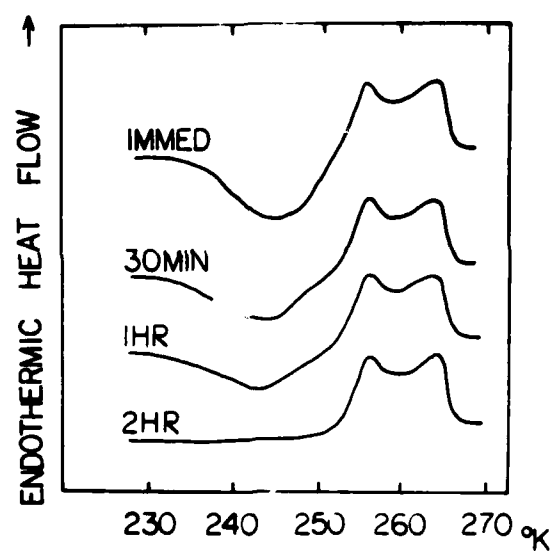


FIGURE 11

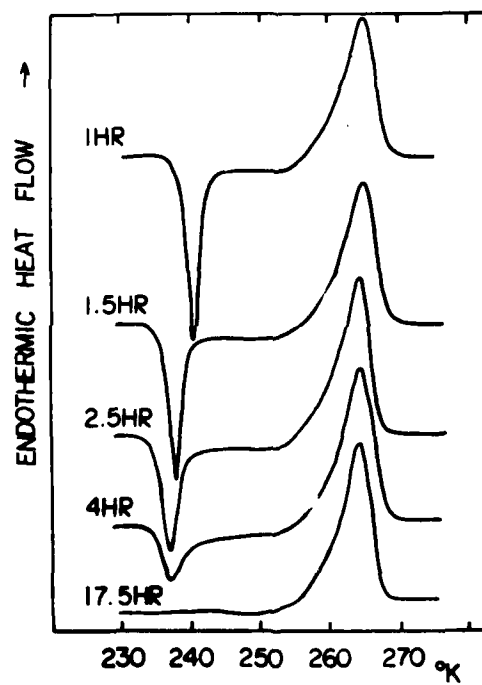


FIGURE 12

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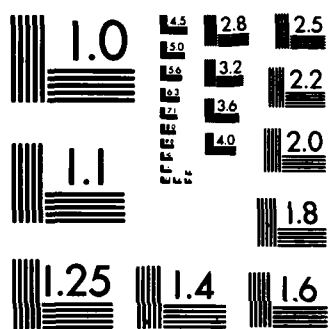
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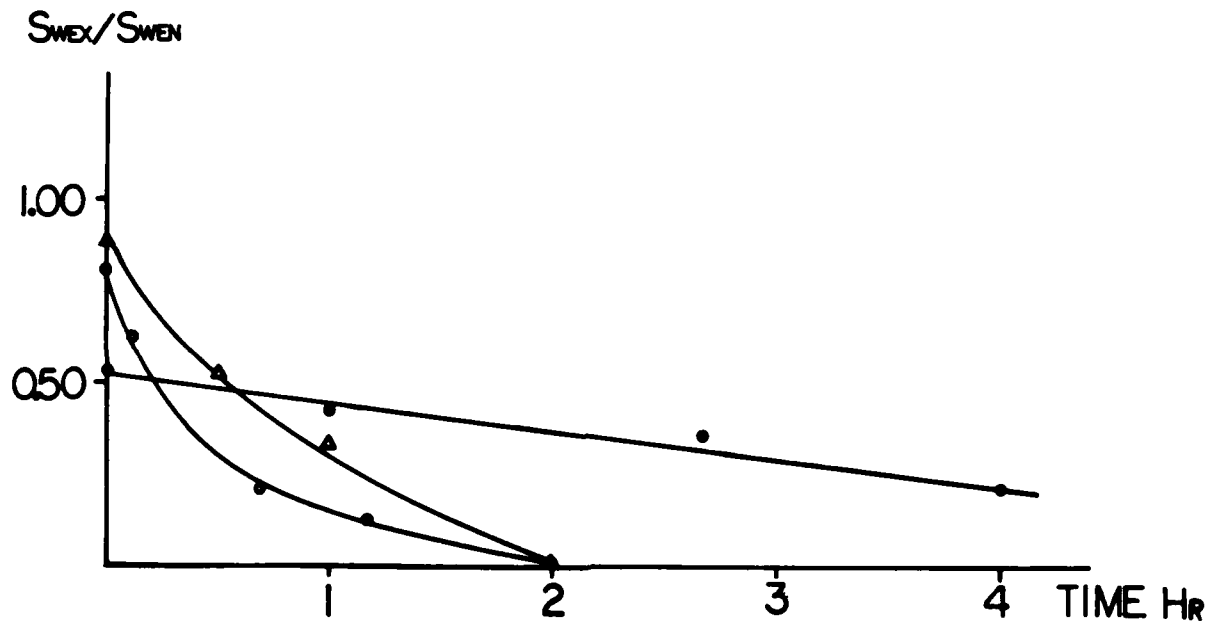


FIGURE 13

ONLY SOLID RED BLOOD CELL GHOSTS TRANSPORT K^+ AND Na^+ AGAINST CONCENTRATION
GRADIENTS: HOLLOW INTACT GHOSTS WITH K^+ - Na^+ ACTIVATED ATPASE DO NOT

by

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Short Title: Ion Transport in RBC Ghosts

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SUMMARY

Human red blood cell ghosts, prepared by simple hypotonic lysis are filled with cytoplasmic proteins. These solid ghosts can transport K^+ and Na^+ against concentration gradient in the presence of ATP. 6

In hollow red cell ghosts prepared by repeated hypotonic lysis followed by high salt wash, this ability to transport K^+ and Na^+ in the presence of ATP is lost even though these hollow ghosts membranes remain intact and are equipped with $Na^+ - K^+$ activated ATPase.

INTRODUCTION

In 1963 Hashimoto and Yoshikawa demonstrated an ATP-dependent Rb^+ uptake of human red cell ghosts against a concentration gradient ^(1,2). Ten years later Freedman demonstrated a similar ATP-dependent net movement of K^+ into red cell ghosts, accompanied by a net loss of Na^+ ^(3,4). Assuming that these red cell ghosts were membrane-enclosed hollow sacs, Freedman concluded that his findings offered support for the membrane pump theory, according to which K^+ accumulation and Na^+ exclusion are due to the activities of postulated pumps in the cell membrane, and against the association-induction hypothesis, according to which K^+ accumulation and Na^+ exclusion reflect primarily properties of the cytoplasmic protein-water system.

A review of the literature revealed that the procedures used for preparing demonstrably hollow ghosts (e.g., Marchesi and Palade ⁽⁵⁾; Dodge et al ⁽⁶⁾) differ from that used by Freedman, which was a modification of the method of Bodeman and Passow ⁽⁷⁾. Therefore, it was not surprising that Ling and Balter ⁽⁸⁾ soon found that EM plates of red cell ghosts prepared by the method of Bodeman and Passow ⁽⁷⁾, and those prepared by the method of Marchesi and Palade ⁽⁵⁾ showed different pictures: Whereas the red cell ghosts prepared by the Marchesi and Palade method, involving an additional step of high salt wash following repeated hypotonic lysis, are indeed primarily intact hollow sacs (see Figure 2); those prepared by the method of Bodeman and Passow were solid bodies. Subsequently Hazlewood and coworkers ⁽⁹⁾ confirmed the findings of Ling and Balter and extended it to include the methods of Garrahan and Glynn ⁽¹⁰⁾ and of Freedman ⁽⁴⁾. All showed solid ghosts.

These findings showed that Freedman's demonstration of K^+ and Na^+ movements against concentration gradients, offered no specific support for the membrane theory, because the observed phenomena can also be readily explained on the basis of the association-induction hypothesis as reflecting properties of the remaining cytoplasmic protein-water system inside the solid ghosts.

The procedures for red cell ghosts preparation described by Marchesi and Palade produces hollow ghosts; from the representative EM picture given by these authors and reproduced here as Fig. 2, the membranes of these hollow sacs remain intact⁽⁵⁾. The intactness of these hollow sacs was further confirmed by the inability of externally added lead phosphate to penetrate into the sacs. Ghosts prepared by their methods including repeated lysing and high salt wash also retain normal activities of K^+ - Na^+ activated ATPase, widely believed among proponents of the membrane pump theory, to be the Na pump itself⁽¹¹⁾.

To probe deeper into the basic mechanism of K^+ accumulation and Na^+ exclusion in living cells, we undertook an investigation to answer three questions:

1. Can we confirm in general the findings of Hashimoto, Yoshikawa, and Freedman? And, in particular, can we confirm Freedman's finding of the ATP-dependent accumulation of K^+ and extrusion of Na^+ from red cell ghosts against concentration gradients?
2. Are red cell ghosts prepared by precisely the same procedure as that used by Freedman hollow?
3. Finally, can the hollow "Marchesi - Palade" ghosts accumulate K^+ and extrude Na^+ against concentration gradients?

The present communication reports the results of investigations aimed at answering these questions.

MATERIALS AND METHODS

All ghost preparations were made from freshly drawn human blood from young adults.

The methods for the preparation of "Type II" ghosts used in Freedman's ion transport studies, to be referred to as "Freedman's ghosts," loading the

ghosts with Na^+ and ATP, resealing, fractionation with a sucrose cushion were entirely as Freedman had described ^(3,4).

"Marchesi-Palade ghosts" were prepared by essentially the method described by these authors ⁽⁵⁾. The modification of the Marchesi-Palade procedure we introduced was to make the treatments less severe than that they employed. Thus instead of 5 mM Tris-HCl and 1 mM EDTA, which Marchesi-Palade used for lysing, our hypotonic lysing solution contained Na_2ATP (5 mM), MgCl_2 (7 mM), L-cystein (1 mM), Tris HCl (10 mM), Na EDTA (0.1 mM), adjusted to pH 6.0 with NaOH at 3° C. This solution was in fact that used by Freedman and shown by Freedman (and ourselves as well, see below) to produce no serious impairment of the ion transport mechanism. After lysing at 1° C (10 min.) the ghosts were exposed for a total of 10 min. (including centrifugation time) at 4° C to a high salt solution containing 0.5 M NaCl, washed twice more in the ATP-containing lysing solution and then resealed by adding to each 100 ml of the ATP-containing lysing solution-ghost suspension, 20 ml of a hypertonic mixture of NaCl (0.5 M), KCl (15 mM), sucrose (2 M).

The procedure for demonstrating K^+ and Na^+ movements, also followed Freedman ^(3,4). Briefly the resealed "Type II" ghosts were incubated at 37° C as a 20% suspension (v/v) in an incubation solution containing the following: NaCl (50 mM), KCl (10 mM), MgCl (2 mM), Tris-HCl (10 mM), $\text{Na}_2\text{H EDTA}$ (0.1 mM), inosine (10 mM), adenosine (10 mM), sucrose (160 mM). The pH of the medium was 7.4 measured at 37° C. At intervals, aliquots of the ghost suspension were taken out, spun in 0.4 ml polypropylene microcentrifuge tubes before assay for K^+ , Na^+ (by atomic absorption spectroscopy) and water contents (by drying at 100° C. in vacuo). Following Freedman ^(3,4), we made no corrections for ions in the extracellular space which in intact red cell pellets usually amounts to 1.5 to 3.0% (Maizels and Remington ⁽¹¹⁾).

For visualization by transmission electron microscopy, pellets of the ghost preparations obtained by centrifugation were fixed in glutaraldehyde, dispersed to avoid layering and non-random sampling errors and then post-fixed in osmium tetroxide, dehydrated through alcohol and propylene oxide, imbedded in Epon, and then stained in uranyl acetate and lead citrate. Essentially the same procedure was used by Marchesi and Palade ⁽⁵⁾ for their EM plate shown as Fig. 2 below.

RESULTS

Are the Red Cell Ghosts Hollow?

Figure 1 shows an EM plate of "Type II" red cell ghosts prepared by the Freedman procedure ("Freedman's ghosts"). Like those prepared by the original method of Bodeman and Passow, these ghosts are also solid. We chose to present a picture of not too low a magnification so that it can be compared with that of Marchesi and Palade ⁽⁵⁾. For other pictures where larger populations of "Freedman's ghosts" were shown, see Hazlewood et al ⁽⁹⁾ whose conclusion Fig. 1 confirms. Since human cells contain little or no polynucleotides, the uranium and lead staining material seen inside the ghosts most likely reveals the presence of substantial amounts of cytoplasmic proteins. In contrast, Fig. 2 reproduced from Marchesi and Palade ⁽⁵⁾ shows intact but hollow "ghosts."

Can the "Freedman Ghosts" Transport K^+ and Na^+ Against Concentration Gradients?

In Figure 3, the K^+ and Na^+ concentrations in the "Freedman ghosts" are expressed as a ratio (ρ -value) to the concentration of the respective ion in the external incubation solution and are shown against the time of incubation. ATP was introduced into the ghosts only during hemolysis and resealing in both cases. The "Freedman ghosts" showed moderate K^+ accumulation and Na^+ exclusion against concentration gradients as Freedman described earlier; the time course of K^+ concentration and that of Na^+ extrusion from the ghosts diverged with time. The lowest level of Na^+ reached was about 60% of that in the external solution. The highest level of K^+ reached in the cell was 30 mM, 6 times higher than that of the K^+ concentration in the surrounding medium which was 5 mM. In normal intact human erythrocytes, the Na^+ concentration is about 16% that in the external solution; the K^+ concentration is about 155 mM, more than 27 times higher than the K^+ concentration in the plasma which is also 5 mM ⁽¹²⁾.

Can the "Marchesi - Palade Ghosts" transport K^+ and Na^+ against concentration gradients?

As shown in Figure 4 the "Marchesi-Palade ghosts" behaved differently under the same experimental conditions. The concentrations of K^+ and Na^+ remained essentially unchanging: there was no active transport of K^+ or Na^+ . When the K^+ and Na^+ concentrations did exhibit minor changes, they rose and fell with time in a parallel manner. These parallel K^+ and Na^+ changes, in contrast to the divergent changes shown in Figure 3 probably reflected minor fluctuation of the water contents of the ghosts. It may be mentioned that we also prepared ghosts according to the procedures of Dodge et al ⁽⁶⁾, a method also known to produce truly hollow ghosts. The results were quite similar to those shown in Figure 4.

DISCUSSION

Confirmation of Freedman's Demonstration of K^+ and Na^+ Movements Against Concentration Gradients in Ghosts that are Solid

This paper is not the first to report that simple hypotonic lysis does not produce hollow ghosts; besides those earlier mentioned ^(8,9), Eric Ponder reached the same conclusion from a totally different approach ⁽¹³⁾ three years after the publication of his authoritative monograph, "Hemolysis and Related Phenomena" in 1948 ⁽¹⁴⁾. Taken together, these findings invalidate Freedman's claim that his observation specifically supported the pump theory since this conclusion was based on a wrong assumption. However, the data shown in Figure 3 do confirm the factual findings of Freedman concerning transport of K^+ and Na^+ against concentration gradients in ghost preparations now known to be not hollow but solid. In a general way these findings of course also confirm the conclusion of Hashimoto and Yoshikawa ⁽¹⁾.

Failure of Pure and Intact Plasma Membrane-enclosed Sac to Pump K^+ and Na^+

Marchesi and Palade showed that the majority of these ghosts prepared by their procedure, which we followed, are not leaky. As mentioned above, in the context of the membrane pump theory, these ghosts (which were subjected to a much milder version of the preparatory procedures of Marchesi and Palade) also possess normal Na pump, i.e., the K^+ - Na^+ activated ATPase as

these authors demonstrated ⁽⁷⁾. It would seem that the necessary conditions for pumping Na^+ out and accumulating K^+ in the ghosts were fulfilled and that active transport of K^+ and Na^+ should occur in the presence of ATP. Yet this was not the case.

Confirmation of the reported K^+ accumulation and Na^+ exclusion in the ghosts now shown to be solid can be compared with the success in demonstrating both K^+ accumulation in and Na^+ exclusion from _____ a muscle-cell preparation whose postulated cell membrane pumps were incapacitated, in part by surgical amputation, and in part by the deprivation of "sinks" or "sources" for the ions involved ^(15,16). These effectively membrane-pump-less open ended cell preparations, called EMOC preparation, demonstrate that the ability to handle K^+ and Na^+ distribution resides not in the cell membrane but in fact within the cytoplasm itself.

On the other hand, the failure of an intact but hollow membrane pump preparation obtained with the "Marchesi - Palade" procedure actively to transport Na^+ or K^+ agrees with a similar failure to demonstrate active transport of K^+ and Na^+ in squid-axon membrane sacs from which the bulk of cytoplasm had been removed ^(17,18). Here the anatomical as well as functional intactness of the axonal membrane was also clearly established, by electron microscopy ⁽¹⁹⁾ and by the full and normal electrical behaviors observed ^(19,20), which according to the membrane pump theory indicate normal membrane and healthy pumps.

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LEGENDS

Figure 1 - Electron micrograph of human red cell ghosts prepared by the method of Freedman ("Freedman Ghosts"). Magnification 18,000x (Plate No. 383).

Figure 2 - Electron micrograph of a representative area in a section through ghost membrane pelleted by high speed centrifugation (100,000 g, 30 min) and fixed in glutaraldehyde- OsO_4). The ghosts appear as empty sacs bounded by continuous unit membranes. Fibrillar material is seen along the inner surfaces of the ghost membranes. Magnification 90,000x. (from Marchesi and Palade, by permission of the J. Cell Biol.)

Figure 3 - Demonstration of active transport of K^+ and Na^+ against concentration gradients in the "Freedman Ghosts." Ordinate represents the ratio of the concentration of K^+ or Na^+ ion in the ghost water over the concentration of the same ion with the incubation media. This ratio is called the ρ -value. Each point is the average of at least four determinations. The diameter of the solid circles (Na^+) and hollow circles (K^+) represent twice the standard errors.

Figure 4 - Demonstration of a lack of active transport of K^+ and Na^+ against concentration gradients in the "Marchesi-Palade ghosts." Ordinate represents the ratio of the concentration of K^+ or Na^+ ion in the ghost water over the concentration of the same ion in the incubation media. This ratio is called the ρ -value. Each point is the average of at least four determinations. The diameter of the solid circles (Na^+) and Hollow circles (K^+) represents twice the standard error.



FIGURE 1



FIGURE 2

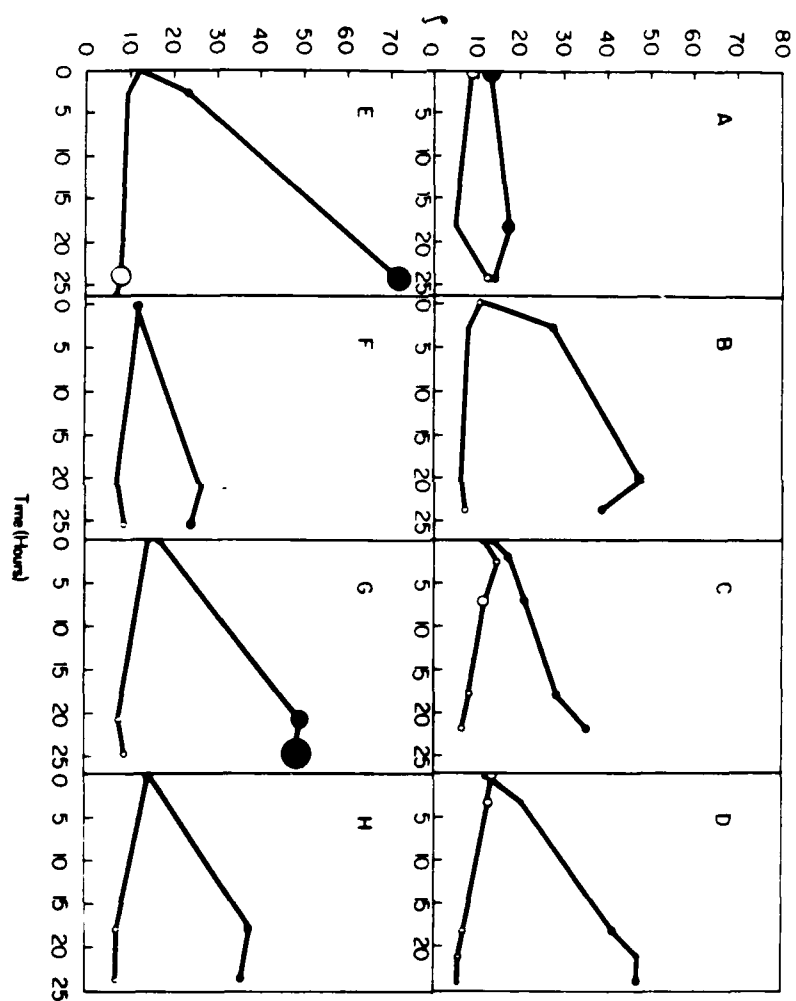


FIGURE 3

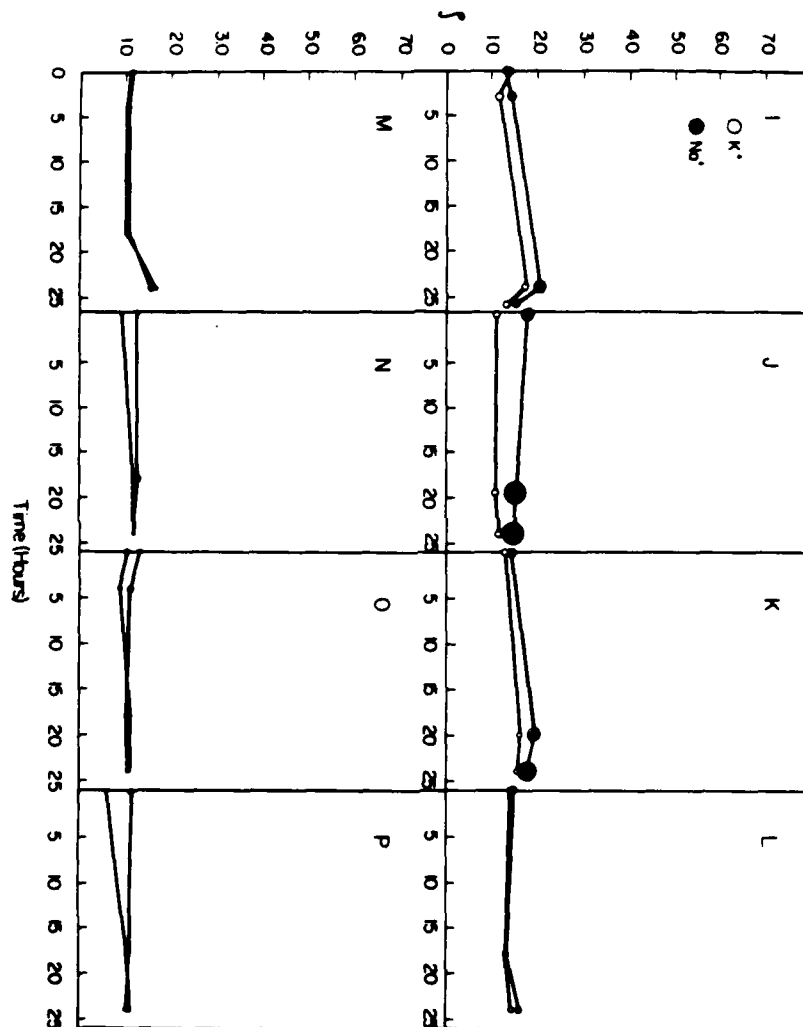


FIGURE 4

The Physical State of Water and K^+ in Living Cells

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From dinosaurs to amoebas all life forms are made up of living cells. Since we now believe that the living world follows the same physical laws that govern the inanimate world the most fundamental question in biology is, "What in physico-chemical terms is a living cell?" To begin with, let us first see what is the chemical composition of these fundamental units of life.

By mass, the largest component of the living cell is water, the next largest component is proteins; by number, the largest component is water again, the next largest component is K^+ . Most living cells are found in an environment in which the largest component is water and the next largest is Na^+ . Since in composition the inside and outside of a living cell resemble each other, the question arises, "What preserves the living cells as discrete units separate from its external environment and maintained unchanged in its chemical composition over long periods of time?" This discreteness and separateness of the cell from its environment is highlighted by the marked segregation of the two chemically similar ions K^+ and Na^+ , one is found at high concentration only within the cell but not outside, while the other is found at high concentration only in the external medium and not in the cells. So to a first approximation, the answer to the question of segregation of K^+ and Na^+ will tell us what are the general principles that determine one of the most crucial properties of the living cell, i.e., its separateness from its environment. Clearly without this basic property, living cells cannot possibly exist.

There are three, and to the best of our knowledge, only three basic mechanisms whereby a substance can be maintained over an indefinitely long period of time at two different levels in contiguous spaces (1): Mechanism I: there is an insurmountable energy barrier separating the spaces; Mechanism II: there is continuous energy-consuming pumping activity; and Mechanism III: there are differences in the physico-chemical environment in the two spaces.

Each one of these mechanisms have been hypothesized to underlie the segregation of K^+ and Na^+ (and other solutes as well). Pfeffer's membrane theory (2) which postulated membranes absolutely impermeant to some solutes was based on Mechanism I; Donnan's theory of membrane equilibrium in which permeant ion distribution was determined by their charges and that of impermeant ions (3) is a variant of Mechanism III. The demonstration that Na^+ , long held as a typical impermeant solute is actually in rapid and constant exchange between the inside and outside of the cell (4, 5) disproved on one hand, absolutely membrane impermeability mechanism (Mechanism I) and on the other hand, it also shows the inadequacy of the Donnan theory of membrane equilibrium, since according to the Donnan theory all monovalent ions of the same electric charge should distribute equally across the cell surface. The remedial Na^+ pump idea to explain the low level of Na^+ , contrary to the prediction of the Donnan membrane theory invokes Mechanism II (6,7).

The membrane-pump theory is the theory almost universally taught as fact in textbooks and serves as the underlying foundation for the great majority of biomedical research.

This wide acceptance is in part due to the ability of the membrane-pump theory at one time to explain the major phenomena of cell physiology: selective solute distribution, selective permeability, volume regulation and osmotic behavior, and cellular electrical potential (8, 9) - all squarely dependent on the validity of the basic tenets of the membrane-pump theory that K^+ and water in the living state exist in a free state much as Na^+ and H_2O are in the external medium. At the time competing theories were still too poorly developed. Methods for testing the alternative theories were also not yet available. But these conditions have changed drastically in the last 30 years and strong evidence indicate that there are serious difficulties with the membrane-pump theory, which includes

theoretical ambiguity and extensive contradictory experimental evidence.

The theoretical ambiguity includes the failure, after forty years, of its proponents to state precisely how many pumps there must be in order to maintain the solute distribution pattern of living cells (10, p. 217; 11 - 13) and to reconcile their total energy needs with the fact that one pump alone, the Na^+ pump, would consume at least 15 to 30 times the total energy available (10, Chapt. 8) - a finding never seriously challenged but twice confirmed (14, 15). Three remedial postulations have been introduced to keep the pump afloat; Ussing's exchange diffusion mechanism (16), Na^+ sequestration in sarcoplasmic reticulum (17), and non-energy consuming Na^+ pump of Glynn (18). All have been experimentally disproven (12, p. 8; 19). At last count in 1968, more than 20 pumps had already been proposed (12, Table 2).

Other experiments that contradict the membrane-pump theory include (i) the failure to demonstrate in cytoplasm-free squid axon membrane sacs, fortified with ATP as energy source, to pump Na^+ out and K^+ in against concentration gradients when these sacs were filled with and bathed in sea water (1), even though by its electrical and other activities the membranes were shown to be perfectly normal; and (ii) in an effectively membrane(pump)-less open ended (EMOC) frog muscle cell preparation, K^+ accumulation and Na^+ exclusion persist (20).

Finally, one comes to the crucial question about the physical state of K^+ and water in living cells. As mentioned above, a key postulate of the membrane-pump theory is that the bulk of intracellular K^+ and water exists in the free state as in a dilute salt solution. It was A. V. Hill who in the thirties produced the widely hailed proof of free K^+ and free H_2O in frog muscle cells that constituted one of the most persuasive sets of evidence (21, 22).

A. V. Hill's influential argument for free K^+ and free H_2O in cells began

with his demonstration that there is no "bound" or "non-solvent" water since the probe molecule urea was shown to be equally distributed between muscle cell water and the external solution. He then measured the vapor pressure of resting muscle and showed it to equal that of an isotonic NaCl solution. Since water is free, this osmotic activity of the muscle cell indicates that the major intracellular cation, K^+ , must be free. However, Hill's argument rests upon the old and inaccurate concept of "bound" water and its assumed (incorrectly) non-solvency towards all solutes including urea. What Hill demonstrated was that this specific theory of bound, or non-solvent water was not right; he did not prove that water was really all free as in a normal 0.1 M NaCl.

Although the membrane-pump theory has dominated the field for so many years, it is by no means the only theory of the living cell. For a full review of other theories, the reader may consult a comprehensive treatise I have just finished writing, "In Search of the Physical Basis of Life" to be published by the Plenum Publishing Corp. of New York in the fall of 1983. For the moment, I shall concentrate on one theory, that presented under the title, the association-induction (AI) hypothesis (1,10,11) which shares a number of common features with Troshin's sorption theory (23).

According to the AI hypothesis the high concentration of K^+ in the resting cell results from the selective adsorption of K^+ (e.g., over Na^+) on anionic β^- and γ -carboxyl groups of cellular proteins (10, 24) and the low level of Na^+ on the other hand, reflects the existence of the bulk of cell water in the state of polarized multilayers due to adsorption on protein chains existing in an extended state (25, 26). The depth of the multilayer is as a rule no more than 10 molecules between chains. What are these matrix proteins remains to be determined but there are evidence that they may include actin (27). The solubility of small solute molecules and solute that can fit into the multilayer matrix distribute

themselves equally between polarized water and normal liquid water with equilibrium distribution coefficient, (or q-value) near or even somewhat exceeding unity; larger molecules have decreasing q-values with increasing molecular size and complexity (12, 28, 29).

The selective K^+ accumulation and Na^+ exclusion depends on the maintenance of the cooperatively associated cell protein-water-ion system at a high energy state when certain key controlling site (cardinal site) on the protein is occupied by the cardinal adsorbent, ATP (1). Removal or hydrolysis of ATP causes the system to assume a more dissociated, lower energy active state in which the ability to selectively adsorb K^+ and exclude Na^+ (as well as sugars and free amino acids) are lost.

According to the AI hypothesis, urea should be equally distributed between polarized water and normal water (26). Thus its demonstrated equal distribution is entirely in harmony with the AI hypothesis and in no way constitutes proof of free cell water. Without proof of free cell water, Hill's arguments for free K^+ also collapse.

New experimental evidence supporting this theory have been gathering rapidly. They include the successful demonstration that proteins with the polypeptide existing in an extended conformation and their backbone NHCO groups directly exposed to bulk-phase water as well as model polymer containing oxygen atoms (as do proteins) at regular intervals with distances between nearest oxygen atoms equal to that of two water diameters polarized multilayers of water. Water so polarized shows decreased solubility for Na^+ , sugars, and free amino acids which are kept at low levels in living cells (26). In contrast when the backbone NHCO groups are locked in α -helical and other intramacromolecular H-bonds, no effect of water solvency occurs (Table 1). These findings confirm the theoretical expectations of the polarized multilayer

theory of cell water, an integral part of the association-induction hypothesis. In this theory it is the existence of alternately positively and negatively charged sites separated by distances of one water diameter apart (as in an extended polypeptide chain) or as a variant, polymer containing oxygen atoms at distances of two water diameters apart that can polarize multilayers of water and water so polarized has reduced translational as well as rotational motion freedom and so are solutes dissolved in this water with a consequent reduction of the entropy of the solute. This lowered entropy of solute is one cause for the reduced solubility of Na^+ and other solutes in the polarized water. Another cause for the reduced level of Na^+ found in this water is the unfavorable enthalpy, since it would take more energy to excavate a hole in the polarized water to accommodate the solute transferred into it than the energy gained in filling the hole left behind in normal water. Both the entropic and enthalpic mechanisms are such that the equilibrium distribution coefficient, or q-value, between polarized water and normal water is near unity for small, spherically symmetrical molecules or molecules that can fit into the polarized water lattice but the q-value decreases with increasing size and complexity of the solute molecules. This is according to the AI hypothesis why urea has a q-value of near unity while hydrated Na^+ , sugars, and amino acids have much lower q-values.

Just as important or perhaps even more so, were the confirmation of the predicted localized adsorption of cell K^+ (or its surrogates Cs^+ and Tl^+) at the edges of the A band and at Z-lines (Fig. 1). The A band and Z-lines in frog muscle cells are the sites where β - and γ -carboxyl groups are concentrated as shown by protein amino acid residue data of myosin which is found only in the A band and from the staining pattern of uranium ion which also binds the β - and γ -carboxyl groups in glutaraldehyde fixed, uranium stained muscle cells (30 to 34). These findings were established unanimously from 3 different laboratories, using a total of 4 different methods (i.e., autoradiography of air dried and on frozen

dried single muscle cells, transmission electron microscopy, dispersive x-ray microprobe analysis, and laser mass spectrometer microprobe analysis (LAMMA)).

While these methods established the localized distribution of K^+ in frog muscle cells, other experiments with intact and EMOC preparation of frog muscle showed the K^+ localization is due to one site-one ion adsorption and not due to existence of K^+ as free counterions, the displacing should be equally effective (35).

The clear establishment of the adsorbed and hence osmotically inactive state of the major intracellular cation, K^+ leaves the balance of intracellular osmotic activity against external isotonic NaCl to the only possible agent remaining, i.e., (matrix) proteins. Because after all, osmotic activity is only a measurement of a lowering of water activity. That multilayer polarization of water lowers water activity is established by the measurement of osmotic activity of the different model polymers which polarize water, including polyvinylpyrrolidone (PVP) and poly(ethylene oxide) (PEO). Due to the large molecular weight (600,000) a 30% PEO solution is only 0.5 mM. Yet this electrically uncharged polymer is able to reduce water activity to equal that brought about by 1 molar sucrose solution (36).

The next question is, "How does the AI hypothesis explain the well-known rules of non-electrolyte permeability relating oil/water distribution coefficients directly to their permeability through the living cell membranes?" Although it is never suggested in the AI hypothesis that the cell surface is identical to any cross-section through the cell, yet in principle and qualitatively, the behavior toward ion and non-electrolyte permeation can well be explained by the cell surface as a two-dimensional version of the three-dimensional cell interior according to the AI hypothesis. The AI hypothesis first points out that the cell membrane resistance is, as a rule, vastly lower than artificial lipid bilayers

($10^8 \Omega/\text{cm}^2$) (37) and often as low as $1 \Omega/\text{cm}^2$ or even lower (38, 39). Thus the conventional picture of a continuous layer of lipids as the foundation of all cell membranes is highly questionable.

Doubts turned very serious when it was discovered (i) that the railway-like structure of the inner membrane of liver mitochondria seen in EM is not materially changed after extraction of 95% of the mitochondria lipids (40) - a finding repeatedly confirmed in studies of other eukaryotic (41) and prokaryotic plasma membranes (42), and (ii) exposure of living cells with high resistance membranes (e.g., squid axon) to various ionophores (e.g., monactin) which specifically enhances K^+ permeability of artificial lipid bilayer membranes has no discernable effect (43). Similar lack of response of the permeability to K^+ in response to valinomycin, monactin, and nonactin was observed in studies on red blood cells, frog ovarian eggs, and frog muscles. These findings and the highly variable lipid contents of plasma membranes analyzed (44) led me to the conclusion that the general permeability barrier could not be uniformly that of a lipid layer. Additional evidence supporting this view came from the studies of Ling, Ochsenfeld and Karreman (45) on the permeability of tritiated water of frog ovarian eggs, in which it was shown that the rate of diffusion of tritiated water is essentially the same through the cytoplasm as through the cell surface membrane. These data suggest that in these cells at least it is the rate at which tritiated water diffuses through polarized water that determines its surface permeability. I then asked the question, "Could polarized water be the seat of semipermeability of living cells?" To answer this question, I compared the rates of permeation of 11 hydroxylic compounds including water at 3 different temperatures through a living cell membrane (inverted frog skin) and through a cellulose acetate membrane, whose "activated" surface layer contains pores of 45 \AA diameters, which is 5 times larger than the diameters of the virtually impermeant sucrose (46). The results of these studies showed not only a good

correlation ($r = +0.96$) but a good correspondence, since both parameters measured are in the same units. The line through the experimental points obtained by the method of least squares as a slope of 0.99. The data offer an answer why pure lipid membrane, as exemplified by olive oil is "anti-semipermeable," being more permeable to ethanol than to water; the normal semipermeable property of phospholipids membranes may be the result of its electrically charged groups and the water polarized by these polarized groups provides the semipermeable properties.

In summary, disproof of the membrane-pump model is about as complete as Phlogiston theory was at the end of the 18th century. There is also now considerable evidence in support of the AI hypothesis according to which neither cell K^+ nor cell water are free as in the membrane-pump theory. Rather both are adsorbed in cells under normal resting state, K^+ singly on α - and γ -carboxyl groups belonging to a large extent to myosin, and water in multilayers, on matrix proteins existing in an extended state. The precise nature of matrix proteins are as yet undetermined though some evidence suggests that actin, tubulin, and other "cytoskeletal" proteins in non-filamentous forms perhaps play significant roles.

Summary

This review compared the critical experimental evidence that led one time to the wide acceptance of the membrane-pump theory of the living cells but has become high equivocal. Other new unequivocal findings strongly contradict this theory; there is not enough energy to operate the pumps; and K^+ , the major intracellular cation, does not exist in a free state; nor does the bulk of cell water exist in a free state as long believed. The review also showed how in the absence of free K^+ , the cell maintains its osmotic balance by means of the same mechanism which maintains a low level of intracellular Na^+ : the multilayer

polarization of cell water. Furthermore, water in this polarized state also functions as the semipermeable barrier conventionally ascribed to lipid layers punctured with rigid pores.

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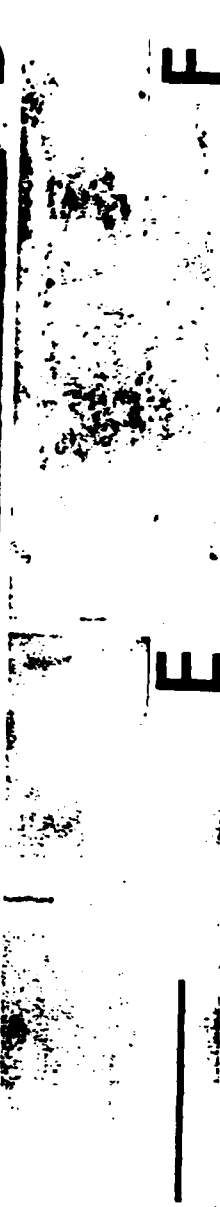
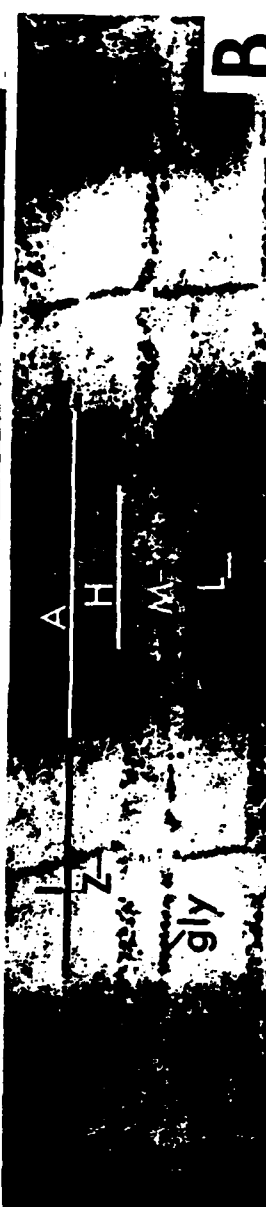
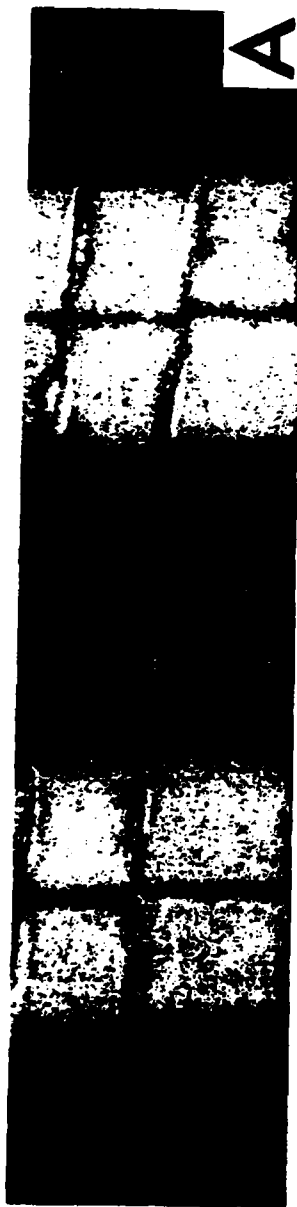
Table 1 "The apparent equilibrium distribution coefficients (ρ -value) of labelled Na^+ between water in protein and polymer-containing solution and normal liquid water determined by equilibrium dialysis"

Temperature was $25 \pm 1^\circ \text{C}$ and test tubes were agitated, except in the experiments of E, which were carried out at $0 \pm 1^\circ \text{C}$ and in which some test tubes, marked Q, were quiescent and unstirred. S represents sacs shaken in test tubes at 30 excursions/min (each excursion spans 1 inch) except the first set (S*) for which agitation was achieved by to-and-fro movement of silicone-rubber coated lead shot within the sacs. The symbols a and b indicate that the media contained initially 1.5 M Na_2SO_4 and 0.5 M Na-citrate respectively. In D, poly(ethylene oxide) (mol. wt. 600,000) was dissolved as a 10% (w/w) solution, and the viscous solution was vigorously stirred before being introduced into dialysis tubing. In E, the quiescent samples contained more water. This higher water content accounts for only a minor part of the difference, as shown by comparison of the 6th and 7th sets of data; even with a larger water content, the ρ -value is lower in the stirred samples (6th). Na was labeled with ^{22}Na and assayed with a Y-counter. (from Ling et al (12)).

Figure 1 Electron micrographs of frog sartorius muscle. (A) Muscle fixed in glutaraldehyde only and stained with uranium by conventional procedure. (B) EM of section of freeze dried Cs^+ -loaded muscle, without chemical fixation or staining. (C) Tl^+ -loaded muscle without chemical fixation or staining. (D) Same as C after exposure of section to moist air, which causes the hitherto even distribution of thallium to form granular deposits in the A band. (E) Section of central portion of B after leaching in distilled water. (F) Normal "K-loaded" muscle. A: from Edelmann, unpublished. B to F: from Edelmann, by permission of Physiol. Chem. Phys.

Group	Polymer	Concentration of medium (M)	Number of assays	Water Content % (mean \pm SE)	ρ -Value (mean \pm SE)
(A)	Albumin (bovine serum)	1.5 a	4	81.9 \pm 0.063	0.973 \pm 0.005
	Albumin (egg)	1.5 a	4	82.1 \pm 0.058	1.000 \pm 0.016
	Chondroitin sulfate	1.5 a	4	84.2 \pm 0.061	1.009 \pm 0.003
	α -Chymotrypsinogen	1.5 a	4	82.7 \pm 0.089	1.004 \pm 0.009
	Fibrinogen	1.5 a	4	82.8 \pm 0.12	1.004 \pm 0.002
	γ -Globulin (bovine)	1.5 a	4	82.9 \pm 0.16	1.004 \pm 0.004
	γ -Globulin (human)	1.5 a	4	83.5 \pm 0.16	1.016 \pm 0.005
	Hemoglobin	1.5 a	4	73.7 \pm 0.073	0.923 \pm 0.006
	β -Lactoglobulin	1.5 a	4	82.6 \pm 0.029	0.991 \pm 0.005
	Lysozyme	1.5 a	4	82.0 \pm 0.085	1.009 \pm 0.005
	Pepsin	1.5 a	4	83.4 \pm 0.11	1.031 \pm 0.006
	Protamine	1.5 a	4	83.9 \pm 0.10	0.990 \pm 0.020
Ribonuclease	1.5 a	4	79.9 \pm 0.19	0.984 \pm 0.006	
<hr/>					
(B)	Gelatin	1.5 a	37	57.0 \pm 1.1	0.537 \pm 0.013
<hr/>					
(C)	PVP	1.5 a	8	61.0 \pm 0.30	0.239 \pm 0.005
<hr/>					
(D)	Poly(ethylene oxide)	0.75 a	5	81.1 \pm 0.34	0.475 \pm 0.009
		0.5 a	5	89.2 \pm 0.06	0.623 \pm 0.011
		0.1 a	5	91.1 \pm 0.162	0.754 \pm 0.015
<hr/>					
(E)	PVP	Q	4	89.9 \pm 0.06	0.955 \pm 0.004
		S*	4	87.2 \pm 0.05	0.865 \pm 0.004
		Q	3	83.3 \pm 0.09	0.768 \pm 0.012
		S	3	81.8 \pm 0.07	0.685 \pm 0.007
		Q	3	67.0 \pm 0.26	0.448 \pm 0.012
		S	3	66.6 \pm 0.006	0.294 \pm 0.008
		Q	3	56.3 \pm 0.87	0.313 \pm 0.025
		S	3	55.0 \pm 1.00	0.220 \pm 0.021

Table 1



The Osmotic Activity of Aqueous Solutions of Several Polymers including Gelatin,
Polyvinylpyrrolidone and Poly(ethylene oxide) Which Reduce the Solubility
of Water for Na^+ , Sugars, and Free Amino Acids

by

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Gelatin, the denatured collagen and a major component of glue, has long interested biologists and chemists due to its many unusual properties. Thomas Graham used gelatin to represent a class of substances which he called colloids (KOLLOID, glue) (Graham, 1861) Katz (1919), Bungenberg de Jong, and colleagues (Holleman et al, 1984), Lloyd and Moran (1934), and others discovered many important properties of water in the gelatin-water system. Recently by making use of a modification of the dialysis technique, which was introduced by Graham also (i.e., the equilibrium dialysis method) Ling and coworkers (1980a, b) presented evidence that some of the outstanding features of gelatin-water system might arise from the extensive interaction with the bulk phase water with the gelatin peptide chains which exist in an extended and thus "exposed" conformation in consequence of its possession of repeating units of the triad glycine, proline and hydroxyproline (Veis, 1964), all well-known helix breakers (Chou and Fasman, 1974). It was shown that water under the influence of gelatin has reduced solubility for Na^+ salts, sucrose and free amino acids. In harmony with this view that it is the extended polypeptide chain that caused this change of the property of water, 13 globular proteins show no or very little effect on water solvency. However, solution of these proteins acquire reduced solubility for Na^+ , sucrose, and glycine when these proteins are denatured by denaturants (i.e., urea, and guanidine HCl) that break the secondary structures but not those that break the tertiary structure (e.g., SDS and n-propanol). Additional support came from the study of electrically neutral (Ling et al, 1980a) simple polymers which like extended polypeptide chains, also possess oxygen atoms at regular intervals roughly equal to twice the diameters of water molecules. The most outstanding among these polymers are polyvinylpyrrolidone (PVP), and poly(ethylene oxide) (PEO).

The ability of water dominated by gelatin, PEO, PVP, etc., partially excluding Na^+ , sucrose, and glycine provide one set of experimental data which agree with the hypothesis that similar exclusion of these solutes from living cells may arise

from bulk-phase cell water existing in a physical state similar to water dominated by gelatin, PVP, and PEO, etc.

Our interest in gelatin, like others before us, is that the normal properties of gelatin may find counterparts in the living cells. Troshin (1966) suggested the living protoplasm and gelatin share the common attributes of coacervates; both partially exclude Na^+ , sugars, and amino acid from its water. I suggested that in a subsidiary hypothesis to the association-induction (AI) hypothesis, that certain as yet unidentified matrix proteins (though actin and tubulin are being considered as candidates) existing throughout the cell, may like gelatin, urea-denatured proteins, PEO, PVP in an extended conformation, exercise a polarizing effect on water molecules beyond the first layer and that in these polarized multilayered water, the solubility of small molecules and molecules that can fit into the multilayer dynamic structure have normal solubility. Due to both, or either enthalpic or entropic reasons the solubility decreases with increasing size and complexities of the molecules or hydrated ions like Na^+ , sugars, and free amino acids (the size rule) (Ling, 1965, 1972; Ling and Sobel, 1975). Confirmation of the multilayer of water involved and the size rule have also been reported (Ling et al, 1980b).

Another aspect of the solute distribution problem is selective solute accumulation beyond that found in the surrounding medium as in the case of K^+ . In the AI hypothesis, this involves selective adsorption on anionic sites (Ling, 1952, 1962; Ling and Ochsenfeld, 1966). In voluntary muscles, these sites are localized primarily on the A band and Z-line (Ling, 1977). These predictions of the AI hypothesis have been confirmed in an unanimous manner by three different laboratories across the world, in West Germany, in Hungary, and in the USA, using a total of four different techniques including autoradiography of air dried (Ling, 1977) and frozen muscle cells (Edelmann, 1981), direct EM visualization of electron dense Cs^+ and Tl^+ stoichiometrically and reversibly displacing K^+ (Edelmann, 1980),

dispersive x-ray microprobe analysis (Edelmann, 1978; Tigyi et al, 1981), and laser mass-spectrometer microprobe analysis (LAMMA) (Edelmann, 1981b).

The question these findings raised is, "Since K^+ is the major cation of the cells, its adsorption and hence osmotic inactivity, what then keeps the cell interior in osmotic equilibrium with an isotonic Ringer solution containing 0.1 M of free Na^+ and Cl^- ?"

Since osmotic activity in fact is an expression of a decrease of the activity of the water present, the question can be restated as follows, "What component in the cell can cause the lowering of the activity of cell water (Ling, 1981) to match that of a Ringer solution, now that we know it cannot be cell K^+ ?" According to the association-induction hypothesis, this component is nothing other than the same "matrix proteins" mentioned above that lower the steady levels of Na^+ , sugars, and free amino acids in the cell water. If this idea is correct, we could expect that water dominated by PEO, PVP, and gelatin at a concentration enough to lower the solvency of water should exhibit osmotic activity far beyond that based on its molar concentration. This report describes results testing this prediction.

Materials and Methods

To measure the osmotic activity of polymer-water system, a Wescor Vapor Pressure Osmometer (Model 5100B, Wescor Inc., Logan, Utah) was used. This small and versatile instrument measured the vapor pressure of the solution in a closed chamber by monitoring the dew point temperature depression (which is a fraction of the vapor pressure) with a precision thermocouple hygrometer. While the instrument was designed for handling solutions of low viscosity, it was found suitable to measure osmotic activity of highly viscous solutions as most of the samples studied were. The main departure was to deposit the sample in the sample holder first and to place the paper sample disc over the sample, which in trials show this modification does not in any way adversely affect the results as verified by

repeated reading over a span of time, this verifying procedure should be taken at intervals especially when new samples of different consistencies are dealt with.

Solution (or gel) of 3 synthetic polymers and one protein (gelatin) were studied. The sources were as follows: Polyvinylpyrrolidone (M.W. 360,000) (PVP-360, Lot 57C-0071) was from Sigma Chemical Co. (St. Louis, Mo.); Poly(ethylene oxide) was a gift of Union Carbide, N.Y.; Polyvinylmethylether (Gantrez M-154) was in part a gift from GAF Corp., N.Y., and in part from a purchased lot from the same source. Gelatin, obtained from Eastman, was from pig skin (Lot A4-G, 1EP 8.7, ash content 0.0340), and from calf skin (Lot B4B, 1EP 4.7, ash content, 0.0290).

Samples of three synthetic polymers contain little ashes; gelatin, though of the highest qualities, did contain considerable ionic contaminants. Dilute solutions (ca. 2%) of all polymers and gelatin was first prepared and then ~~ex-~~haustively dialyzed until ashes prepared from the polymer solution when dissolved in dilute HCl yields negligible additional osmotic activities. The dialyzed solution while still in the dialysis sacs were then dried by either being placed in front of a fan (40° C) or packed in a dry dust-free silica gel (Davidson, mesh size 6-16). Great care was taken not to let the polymer dry unevenly by frequently "milking" and squeezing the drying sample toward one end of the dialysis tubing. This method permits preparation of homogenous samples of very high polymer contents, which was individually assayed by oven-drying at appropriate temperature (100° C for PVP, PVME, and gelatin, 80° C for PEO).

Results

Figure 1 shows the osmotic activity of a solution of exhaustively dialyzed gelatin, where the osmotic activity is expressed in units of OsMolal and the gelatin concentration in percentage (W/V). For comparison data from hemoglobin are also presented. Six experimental points from the work of Adair (Adair, 1928;

Adair and Robinson, 1930) are also included which in general agree with our own hemoglobin data which reached a higher concentration of 50%. Comparing with the hemoglobin data, the osmotic activity of gelatin is many times higher. At the higher concentration range neither the osmotic activity of gelatin nor that of hemoglobin is commensurate with the molar concentration of gelatin and hemoglobin present. Thus a 50% hemoglobin solution is roughly $500/6.7 \times 10^4 = 7.45 \text{ mM}$ while the osmotic activity corresponds to a concentration of 450 mM. The molecular weight of gelatin (denatured collagen) is less clearly defined as it contains fractions with molecular weight as high as 10^6 (see Stainsky, 1977). The molecular weight of the α -chain of collagen is close to 90,000 (Piez, 1967). Using a value as low as this, the molar concentration of a 50% gelatin solution is only $\frac{500}{9 \times 10^4} = 5.5 \text{ mM}$ compared to the measured value of 2240 mM! An equally remarkable of the gelatin curve is its pronounced sigmoid shape which indicates that the osmotic activity, while higher at a lower range, abruptly increases when gelatin reaches 45%.

Figures 2 and 3 show qualitatively similar curves for solutions of polyvinylpyrrolidone (PVP) and poly(ethylene oxide) (PEO). Quantitatively, PEO demonstrates the extensive increase of osmotic activity at similar concentrations even though the average molecular weight of PEO (600,000) is higher than that of PVP (360,000) or gelatin. Indeed a 40% PEO has a molar (or molal) concentration of only $\frac{400}{600,000} = 0.67 \text{ mM}$ yet the measured similarity is 2620 mM which is nearly 4000 times higher. Similarly at a 55% concentration, the molar concentration of PVP is only 1.53 mM while the measured osmolarity is 3300 mM or 2160 times higher.

We also studied polyvinylmethylether (PVME) solutions but decided that the observed data are not worthy of publication since it reflects a peculiar kind of artifacts due to the tendency of PVME to separate out into denser phase (coacervate) and lighter phase (dilute PVME solution) as room temperature varied in our laboratory. The persistent presence of droplets of dilute PVME in a denser phase of concentrated PVME makes the measurement of PVME concentration-dependent osmolality reflecting more

the property of these droplets with high water activity rather than the low-water activity dense PVME phase.

The PVP and PEO data shown in Figures 2 and 3 respectively are plotted in a different manner in Figures 4 and 5. Here the ordinate represents the measured osmotic pressure (π) in units of dm H₂O divided by the polymer concentration C_2 in grams per liter of solution. The abscissa represents the polymer concentration C . This plot is, of course, based on the theory (see Tombs and Peacocke, 1974)

$$\frac{\pi}{C_2} = RT \frac{\bar{V}_1^0}{\bar{V}_1} \left[\frac{1}{M_2} + BC_2 + CC_2^2 + DC_2^3 + \dots \right] \quad (1)$$

where R, T have the usual meanings. \bar{V}_1^0 is the volume per mole of pure solvent and \bar{V}_1 is the partial molar volume of the solvent in the polymer solutions. M_2 is the molecular weight of the macromolecule in units of gmol^{-1} . B, C , and D are the second, third, and fourth virial coefficients in units of mol l g^{-2} , $\text{mol l}^2 \text{g}^{-3}$, and $\text{mol l}^3 \text{g}^{-4}$ respectively. However, following tradition the virial coefficients tabulated from these data are given in units of mol m/g^{-2} etc. (Table 1).

The most outstanding features of these plots are the steep curvature and hence very large 3rd or even 4th virial coefficients are required to describe the data. Like in all curve fitting, the values assigned are somewhat arbitrary, especially the second virial coefficient term in cases where a large, higher order term dominates.

Discussion

The most outstanding observation recorded here is the enormous osmotic activity of the aqueous solutes of the three polymers, two neutral (PEO and PVP) and one charged (gelatin) far beyond the molar concentrations of the polymer present. At face value these model studies support the theory of the osmotic effect in living cells due to extended protein chains. However, some contaminants in the form of oxidation reaction may give rise to, say, carboxyl groups and

and their counterions and increase the total osmotic activity beyond that of the originally neutral polymer. Our analysis of the Na contents of PVP and PEO samples first equilibrated in 0.1 M NaCl and then exhaustively dialyzed in distilled water made somewhat alkaline with the addition of NaOH revealed a total Na content of about 5 minimolar concentration in a 40% polymer solution. This is too trivial to make any significant difference. A second source of possible error is our calculated molar concentration of the polymers is the heterodisperse molecular weights. However in dilute solution form the polymer has been exhaustively dialyzed with a dialysis tubing with a molecular cut-off point of about 12,000 daltons. Thus, even if the M.W. of all the polymers studied were 12,000, a 40% solution would still be only $\frac{400}{12,000} = 30 \text{ mM}$, which is far from the recorded osmotic activity of more than 1000 milliosmolar. Thus heterodisperse M.W. could not be the cause of high osmotic activities observed.

Finally the sigmoid-shaped curves, showing an abrupt increase of osmotic activity at around 30 to 40% rules out these and other possible contaminants as the cause of the erroneous osmotic activity at higher concentration since such contaminants as a component of the polymer should linearly increase with polymer concentration. Having eliminated contaminants in the osmotic activity observed and due to their enormous molecular weights, I conclude that gelatin, PVP and PEO which have been shown to have the power of reducing the solubility of water for Na^+ salts, sugar, and free amino acid, do indeed have strong effect reducing the activity of water especially when the polymer reaches a certain concentration.

These general patterns of behavior are shared by the solutions of the two synthetic polymer PEO and PVP and that of gelatin but not of hemoglobin, a native globular protein.

The Structural and Concentration Requirements of the Osmotic Effect of Polymers

The minimal structural requirement for the polymer to have the pro-

nounced water activity reducing effect are the same as those as that producing the reduction of solubility for Na^+ , sucrose, and glycine: the possession of oxygen atoms at regular intervals about 2-water diameters apart and that these oxygen atoms must be freely exposed to the bulk phase water and not locked in α -helical or other intra- or intermacromolecular H-bonds (Ling et al, 1980b). Thus globular hemoglobin does not have the effects seen in gelatin.

The present findings thus provide another set of evidence that the usual property of gelatin and hence Graham's colloid may reside in the powerful effect of its extended ——— chain to reduce the activity of water in its vicinity.

While Figure 1 clearly shows that at all concentration ranges, gelatin has much greater osmotic or water-activity-depressing effect than hemoglobin, it is when gelatin reaches a certain critical concentration at about 45% that the effect becomes suddenly very pronounced. This type of behavior, in appearance, at least reminds one of the sigmoid-shaped oxygen uptake curve of hemoglobin and is generally acknowledged to be due to cooperative interaction among the heme-sites on which oxygen molecules are complexed; the binding of one oxygen molecule enhances the affinity of other sites for more oxygen. In our present case, what we see is that as the polymer concentration increases its effects on the water activity also demonstrates characteristics of cooperative behavior. Let us examine what could be the basis of this phenomenon. Let us focus our attention on PEO first.

Being simply repeating units of $(-\text{CH}_2\text{CH}_2-\text{O}-)_n$, this polymer has no side chains and the only seat of direct interaction with water are the oxygen atoms. Therefore the effect of increasing PEO concentration on water activity could only be due to synergistic effect of water-activity reducing effect produced by one oxygen atom and those of other ethylene oxide oxygen atoms on other chains in the vicinity. The sharp increase of water activity reducing effect may thus be due to the enhancement of the propagated polarization (or induction) and the rotational

immobilization produced nearby ethylene oxide oxygen atoms on nearby chains when the average chain-to-chain distance decreases to a point that the propagated inordinate effect mediated through the water molecules can be strong enough to affect water molecules already acted on by neighboring chains as illustrated in Figure 6.

Since gelatin, PVP, and PEO all show highly similar sigmoid shaped curve in their water activity reducing action and the only H-bonding groups they share are the regularly and suitably separated oxygen atoms on the chain, these models together support the view that certain extended protein chains in living cells may also be responsible to the reduction of water activity to match that of that in the external medium of sea water, plasma, etc., which owe their water-reducing effects to free ions present.

The Relationship Between Water-Activity Reduction and Water-Solvency Reduction

Figure 7 plots the ρ -value for Na^+ (and Mg^{++}) obtained mostly ~~already~~ published data (Ling et al,) but include some new data. Note that a 40% PVP solution the ρ -value for Mg^{++} has dropped to less than 0.1. That is, at least 90% of the water has been so profoundly affected by the PVP that it has lost all its solubility for Mg^{++} . In fact, the exclusion very unlikely could be absolute. Thus it is more reasonable to conclude that all the water has been profoundly affected in its solvency by PVP.

Unfortunately the PVP concentration expressed here in Fig. 7 as well as those of gelatin and PEO cannot be directly compared with those of Figures 1 to 3. The osmotic activity measurements were made on the basis of pure water-polymer systems while that shown in Figure 7 contains besides water, and polymers, also high concentrations of Na citrate. Indeed it was by varying the concentration of these salts that polymer-water system with different water contents were created. (This subject of swelling-shrinkage of polymer-water system will be fully described in another article). Nevertheless, the water activity reduction effect and the solvency reduc-

tion effects show close parallel behaviors including sharp changes at certain polymer concentration and the increasing effectiveness of the three polymers studied: gelatin < PVP < PEO.

One of the reasons previously given for the greater solvency reduction effect of PEO than the two other polymers is its extreme simplicity of structure and inability to form hydrophobic, interchain H-bonds as in the formation of "collagen folds" in the case of gelatins.

The parallel behavior between the water activity reducing effect and the solvency reduction effect of these oxygen-containing polymers are to be expected from the proposed mechanism of both phenomena. The water activity reduction is most likely the consequence of polarization and immobilization by the propagated polarization emanating from the oxygen atoms and the reduction of in particular the rotational "partition functions" ($P_{\text{rot}}^{\text{H}_2\text{O}}$) in statistical mechanical terms. Such a reduction of $P_{\text{rot}}^{\text{H}_2\text{O}}$ reduces the vapor pressure of the water, which was in fact what we observed with the vapor-pressure osmometer used.

The solvency reduction effect has also been explained in terms of a rotational partition fraction reduction, of the large, complex solute molecules or hydrated ions ($P_{\text{rot}}^{\text{solute}}$) (Ling, 1965; 1972). However reduction of $P_{\text{rot}}^{\text{solute}}$ is the consequence of the reduction of $P_{\text{rot}}^{\text{H}_2\text{O}}$ in the same sense that a motional freedom of a butterfly (solute) is restricted when it is caught by the web of immobilized spider web (water) can be cited for liver mitochondria inner matrix. Thus the polymer concentration range needed to produce a marked effect on water activity and solvency, when consideration has been given to the marked enhancement due to fine organization of the matrix protein chains plus the large amount of space-filling globular proteins, like hemoglobin, which by itself in its globular form begin to have its own water-activity reducing effect (see Fig. 1) at concentration comparable to that found in red blood cells. Thus in general, one may say that with the limitation inherent in any model system studies, these findings are in full harmony with the theory of the living cells in regard to both the mechanism for

maintaining low Na^+ , Mg^{++} , and other solutes at low level without continual expenditure of energy and in providing the basis of understanding why cells without free K^+ can nevertheless be in osmotic equilibrium with external medium containing isotonic concentration of free sodium and chloride ions.

Comparison with Living Cells

The concentration of polymers needed to produce a pronounced effect on water activity and on solvency are as a rule quite high (e.g., 40-50%). Can this system be compared with that of the living cells? The answer is two-fold.

First, according to the polarized multilayer hypothesis of cell water, the maximum effect on water polarization and solvency reduction occurs when the extended "matrix protein" chains are fully extended and are organized in a regular array to be expected for the highly organized cell. All the model systems studied are obviously far from this situation being certainly more like a tangled "mess." In support of this expected effect of chain orientation, Ling et al. have shown the ρ_{Na} for PVP-water system decreases with stirring (Ling et al, 1980a, b). These results are in full accord with earlier report of Woessner and Snowden (1973) who gave NMR evidence for increased water structuring as a result of the stirring of another polymer-water system (Kelzan^(R)).

The second point is living cells contain 20 to 30% proteins but some cells (e.g., human erythrocytes) contain as much as 40% proteins. The question is, if the protein chains are indeed fully extended, how would the change of the content of these proteins affect the number of water molecules found between the nearest neighboring chains? For this a simple calculation will reveal some very interesting insights. Thus if one liter of cells contains n grams of proteins, we can assume an average amino acid residue weight of 112 (see Ling, 1962, p. 48), an Avogadro's number of 6.06×10^{23} , a peptide linkage length of 3.5×10^{-8} cm, then the total length of the polypeptide chains added together would equal $\frac{n}{112} \times 6.06 \times 10^{23} \times 3.5 \times 10^{-8} = 1.89 \times 10^{14} n$ cm. Cut into 10 cm long filaments, these

filaments, uniformly distributed, in a $10 \times 10 \times 10$ cm cube, there would be $\frac{1.89 \times 10^{13}}{n}$ or $4.34 \times 10^6 \frac{1}{n}$ filaments to each side and the distances between each nearest neighboring filaments would be $\frac{10}{4.34 \times 10^6 \frac{1}{n}} = 2.30 \times 10^{-6} \frac{1}{n}$ cm.

Figure 8 shows a plot of the percentage of proteins against the distance (right ordinate) and the number of water molecules between each pair of nearest neighboring chains (left ordinate) after assuming a diameter of 3 \AA for each water molecule.

Of course, these calculations assume all the proteins to be in an extended conformation while in truth much of the protein must be in the globular form. To offset the effect of reduced concentration of total extended proteins, is the space occupied by the proteins themselves which can be considerable at the high end of the protein concentration.

The most significant point that this figure demonstrates is how little does the number of water molecules between the protein filaments change with protein concentration. Thus an 8-fold increase of protein contents from 10% to 80% protein has only increased the distance by 2.8 times.

Another important feature shown is that even at a protein concentration as low as 10%, there are less than 8 molecules between a pair of nearest neighboring chain.

This single illustration of the square root relation also raises another question, "If the distance between the chains and hence the number of water molecules spanning two protein chains are so little affected by the protein concentration, why should there be such abrupt change of water activity when gelatin, PVP, and PEO concentration rises from 30 to 50% as the data of Figure 1 to 3 show?"

I believe in part the answer may lie in the cooperative nature of the induced water-to-water interaction and in part, due to the heterogeneous distribution of the polymers in the solutions. Large cluster of tangled polymer chains creates large holes where chain to chain distances are much larger than the calculated average. In agreement, the most likely candidate for forming each chain-to-chain

interaction, gelatin has to reach the highest concentration before reaching the half-way point in the water activity as well as solvency reduction effect. PEO which has the least tendency to form chain-to-chain bonds, reaches its halfway points at the lowest concentration.

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Legends

Figure 1. Osmolality of gelatin and hemoglobin at varying concentrations.

Osmolality are given in Osmolal concentration. Protein concentrations are in % (wt/wt). Each point is the average of at least 4 independent determinations and the distance between horizontal bars are twice the standard errors. Six extra points on the hemoglobin curves shown as X's are taken from Adair's data (see text).

Figure 2. Osmolality of polyvinylpyrrolidone (PVP) solution at varying concentrations.

Details are the same as in Figure 1.

Figure 3. Osmolality of poly(ethylene oxide) (PEO) solution at varying concentrations.

Details are the same as in Figure 1.

Figure 4. Plot of π/C against C in PVP solutions.

Osmotic pressure, π , is in units of cm of H_2O C in grams of polymer per liter. Data are the same as in Figure 2, except that standard error bars are not represented. The solid line going through the experimental points were based on Equation 1. Values of virial coefficients chosen to fit the data are given in Table 1. For comparison the hemoglobin data of our own and from Adair are also shown.

Figure 5. π/C vs. C plots of PEO solutions.

Details are similar to those described in Figure 4. Value of virial coefficients are those given in Table 1. For comparison hemoglobin data of our own and from Adair are also shown.

Figure 6. Plots of apparent equilibrium distribution coefficients of Na^+ salts (ρ -value) of polymer water system against polymer concentration given as % (w/w). New composite plots from data mostly old (but some new) given in different ways of presentation earlier (Ling, 1980a,b). ρ values were obtained by equilibrium dialysis of radioactive labelled- Na^+ in

Na citrate or sulfate solution of high concentration (0.1 M to 1.5 M).

Figure 7. Diagrammatic illustration of the effect of decreasing distance between protein chains or model polished glass surfaces. N represents negatively charged sites and P positively charged sites. NP-NP system represents two juxtaposed surfaces (e.g., polished glass) containing N and P sites at regular spacing like a checkerboard. NP-NP-NP system represents equivalent matrix of linear chains carrying N and P sites at regular intervals separated from each other by distances roughly that of one water diameter. N and P may represent the CO and NH groups of an extended protein chain. PEO and PVP have no proton-donating group and are referred to as NO-NO-NO system where O represents vacant sites and therefore the N to N distance has to equal 2 water-diameters apart.

Figure 8. The theoretical distances both in Ångstrom units (right ordinate) and in number of water molecules between nearest neighboring (fully extended) protein chains in hypothetical case when the entire protein contents (abscissa, in % (w/w) are in the fully extended state and occupy no space.

	B <u>(mol.ml.g⁻²)</u>	C <u>(mol.ml.⁻²g⁻³)</u>	D <u>(mol.ml.⁻³g⁻⁴)</u>
PEO	5.54×10^{-2}	2.17×10^{-1}	-
PVP	1.25×10^{-3}	-	3.28×10^{-2}
Gelatin	3.29×10^{-2}	5.15×10^{-2}	-
Hemoglobin	-	-	1.5×10^{-3}

Table 1

Virial coefficients from the measured osmotic properties of aqueous systems of gelatin, PVP, and PEO

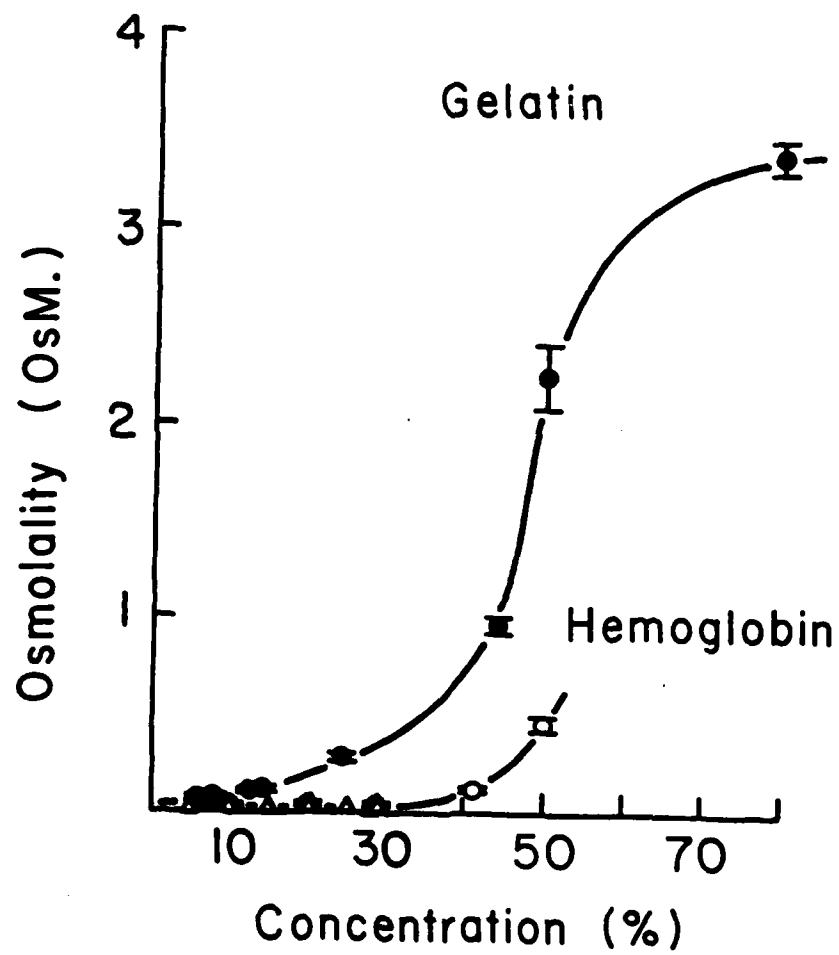


FIGURE 1

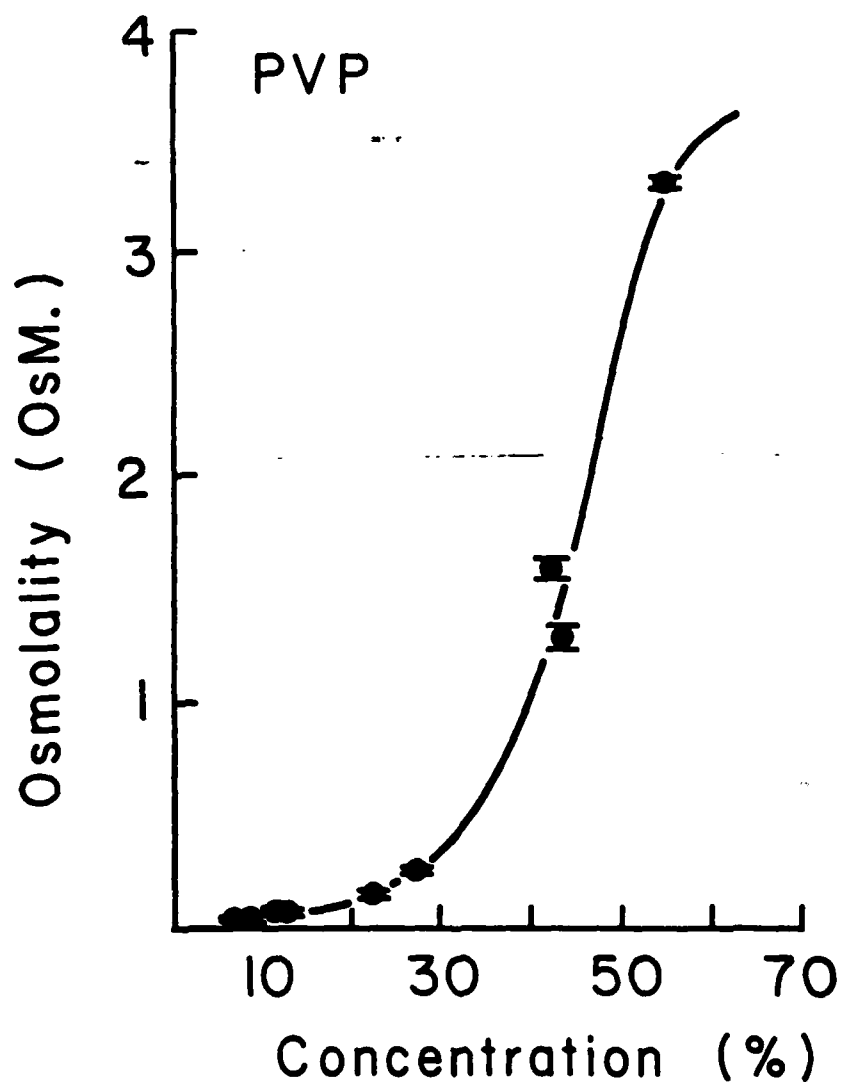


FIGURE 2

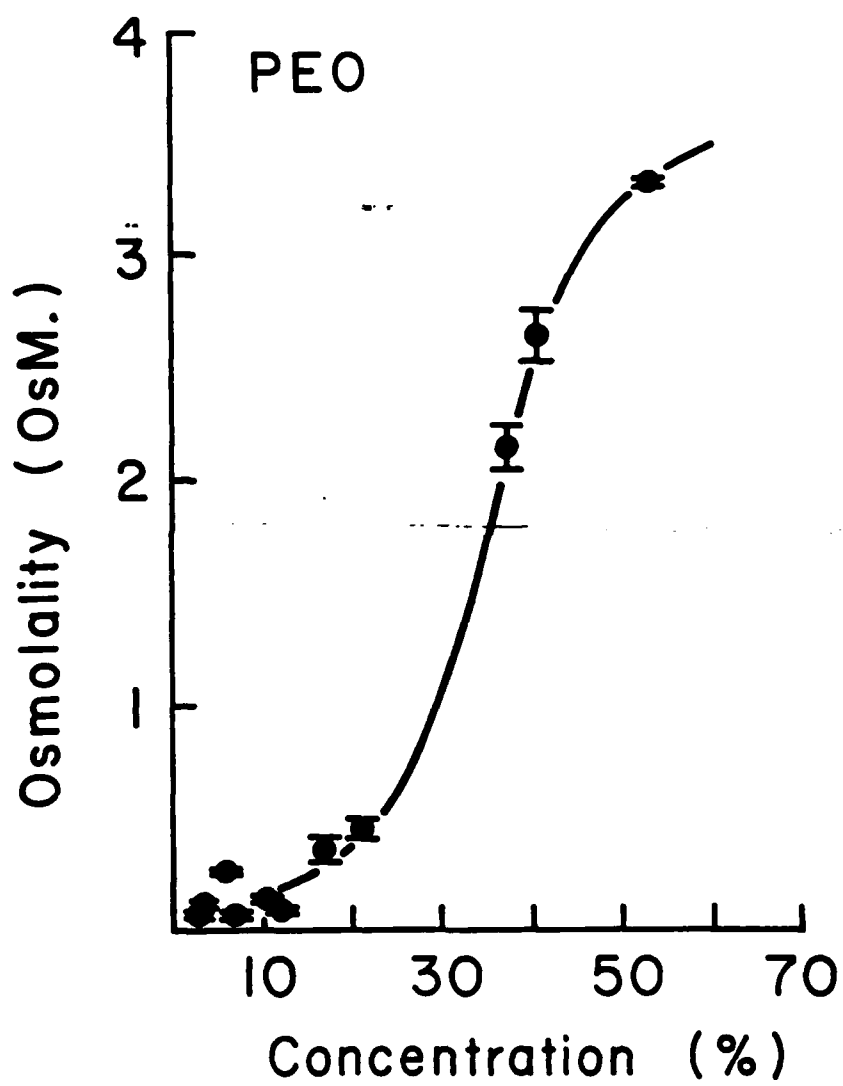


FIGURE 3

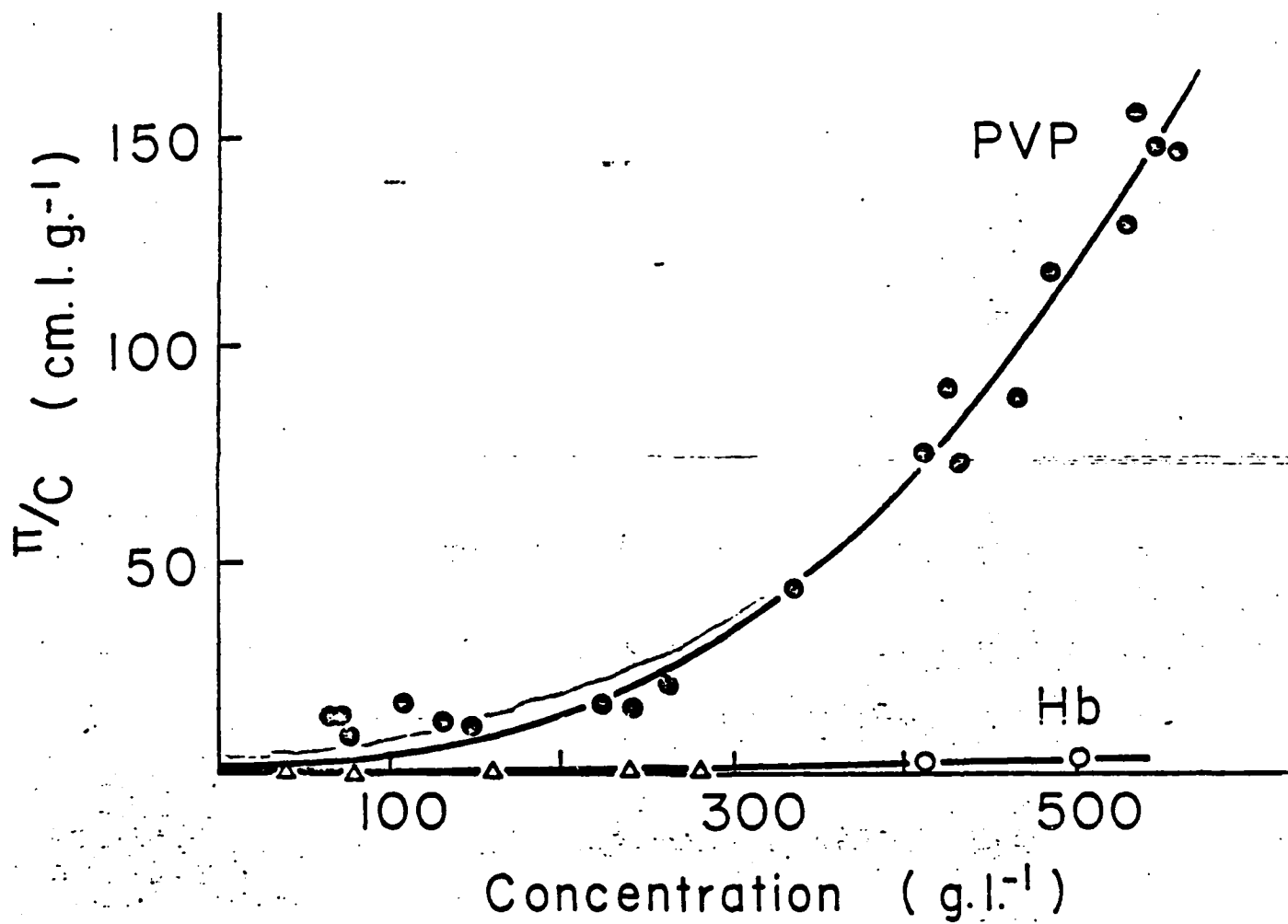


FIGURE 4

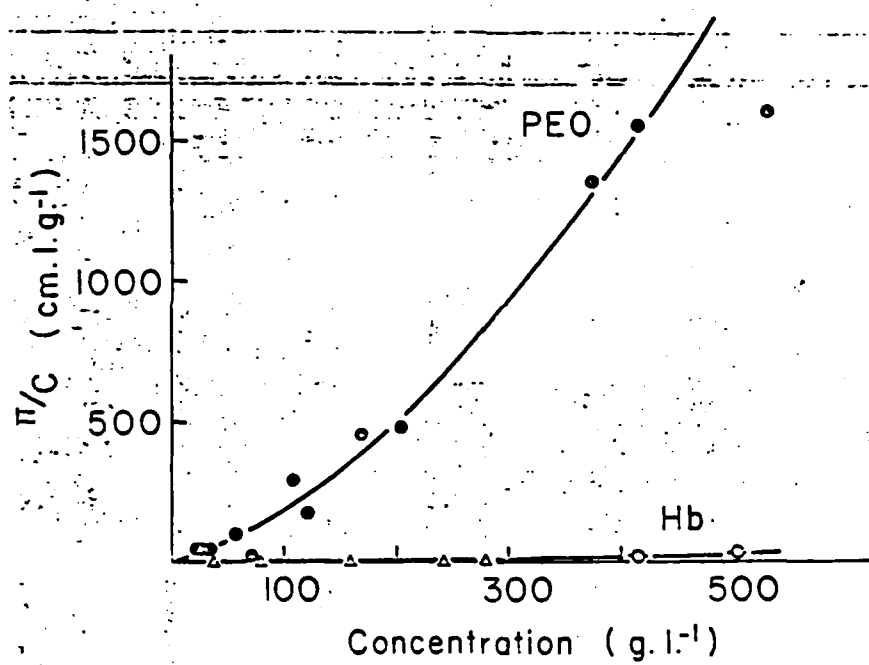


FIGURE 5

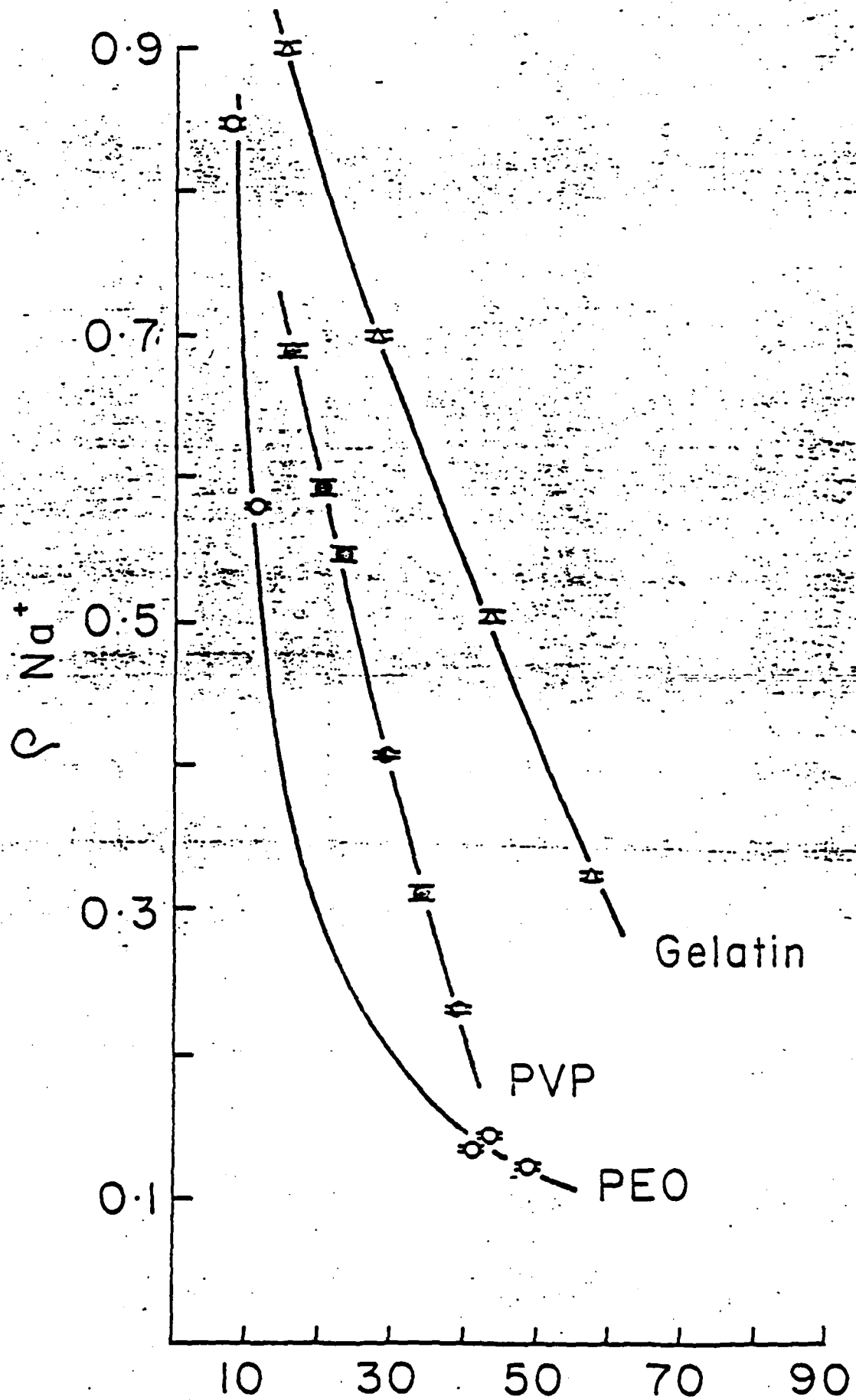


FIGURE 6

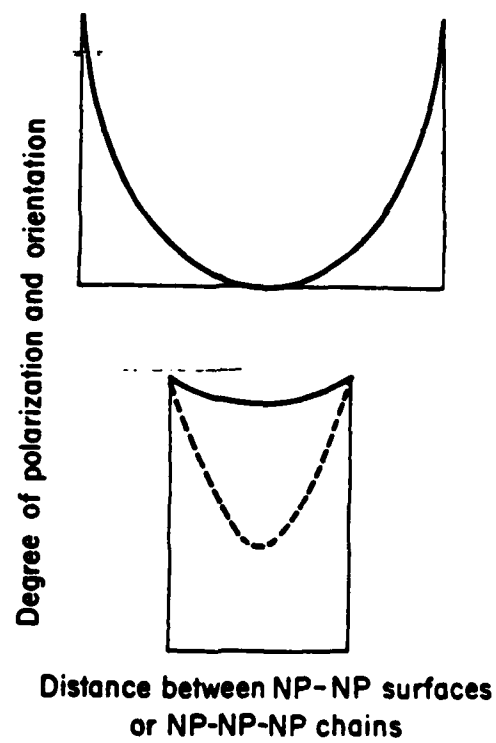


FIGURE 7

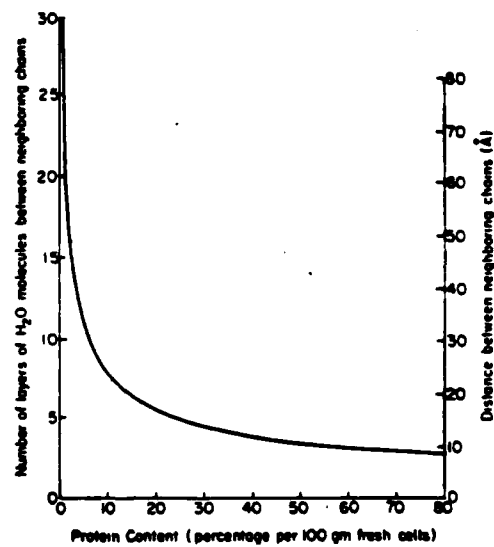


FIGURE 8

COOPERATIVE INTERACTION AMONG CELL SURFACE SITES: FURTHER EVIDENCE IN
SUPPORT OF THE SURFACE ADSORPTION THEORY OF CELLULAR ELECTRICAL POTENTIAL

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INTRODUCTION

Moritz Traub's copper ferrocyanide precipitation membrane (Traube, 1867), with its nearly perfect semipermeable properties, provided the foundation both for Pfeffer's membrane theory of osmotic and solute-distribution properties of living cells (Pfeffer, 1877) and for Ostwald's suggestion of a membrane origin of cellular electrical potentials (Ostwald, 1890). Ostwald's suggestion was developed first into Bernstein's "membrane theory" (Bernstein, 1902) and then into the "ionic theory" of Hodgkin and Katz (the HKI theory) (Hodgkin and Katz, 1949). These and subsequent workers considered membrane permeability the key parameter determining cellular electrical potentials. The relations between the magnitude of the potential and the temperature and between the potential and the external K^+ and Na^+ concentrations have been predicted according to this theory and repeatedly verified. However, a recent survey shows that a great deal of experimental evidence has been collected in the last 25 years; some of them confirm and others contradict the HKI theory (Ling, 1979, 1982). In the same review it was pointed out that the evidence in favor of as well as against the HKI theory together support the surface adsorption theory of cellular electrical potential (the SA theory). This theory is an integral part of the association-induction hypothesis (the AI hypothesis) (Ling, 1962; 1967a,b; 1978; 1982).

According to the SA theory, cellular resting potential bears no direct relation to membrane ion permeability but is related to adsorption of cations on anionic sites, primarily the β - and γ -carboxyl groups of proteins, on the outer cell surface.

In its simplest form, the SA theory of the resting potential (ψ) can be written as

$$\psi = \text{constant} - \frac{RT}{F} \ln \left\{ \tilde{K}_K [K^+]_{\text{ex}} + \tilde{K}_{Na} [Na^+]_{\text{ex}} \right\}, \quad (1)$$

where R and F are the gas and Faraday constant, respectively; T is the absolute temperature; $[K^+]_{\text{ex}}$ and $[Na^+]_{\text{ex}}$ are the external K^+ and Na^+ concentrations; and \tilde{K}_K and \tilde{K}_{Na} are the respective adsorption constants of these ions on the surface anionic sites. It was pointed out that Equation 1 predicts no relationship between ψ and variables (e.g., the cytoplasm K^+ level) that have not been experimentally verified (Ling, 1978).

Equation 1 can be put into a more general form:

$$\psi = \text{constant} - \frac{RT}{F} \ln \sum_{i=1}^n \tilde{K}_i [P_i^+]_{\text{ex}}, \quad (2)$$

where $[P_i^+]_{\text{ex}}$ is the external concentration of the i th monovalent cation among a total of n kinds. \tilde{K}_i is the adsorption constant of the i th species.

More recently, a refined version of the SA theory was presented (Ling, 1979) in which the surface adsorption sites for K^+ or Na^+ are no longer considered to be independent of one another but show an autocoperative interaction, similar to that demonstrated for the bulk-phase adsorption of K^+ and Na^+ in a variety of living cells, (Ling, 1966, Ling and Bohr, 1970; Jones, 1970; Karreman, 1972; Gulati, 1973; Negendank and Karreman, 1978). The equation for the resting potential in this newer version of the SA theory is as follows

$$\psi = \text{constant} + \frac{RT}{F} \ln \frac{1}{[K^+]_{\text{ex}}} \left\{ 1 + \frac{\xi - 1}{\sqrt{(\xi - 1)^2 + 4\theta\xi}} \right\}, \quad (3)$$

where

$$\theta = \exp(\psi/RT), \quad (4)$$

where $-V/2$ is the nearest neighbor interaction energy and

$$\xi = \frac{[K^+]_{ex}}{[Na^+]_{ex}} \cdot K_{Na \rightarrow K}^{oo} \quad (5)$$

$K_{Na \rightarrow K}^{oo}$ is the intrinsic equilibrium constant in the $Na \rightarrow K$ exchange adsorption.

This paper reports experimental studies designed to test the SA theory in general and predictions of Equation 3 in particular.

MATERIALS AND METHODS

We used isolated sartorius muscles of Northern American leopard frog (Rana pipiens pipiens, Schreber) from Vermont. The technique of measuring the resting potential of single muscle fibers was essentially the same as that described by Ling and Gerard (Ling and Gerard, 1950).

The basic Ringer solution contained the following ingredients: 2.5 mM K^+ , 100 mM Na^+ , 1.0 mM Ca^{++} , 1.2 mM Mg^{++} , 86.7 mM Cl^- , 15.7 mM HCO_3^- , 2.7 mM PO_4 , 0.1 mM NO_3^- , and 23.5 mM glucose. In addition it contained 14 vitamins and 21 amino acids as well as penicillin (0.1 mg/ml) and streptomycin (0.1 mg/ml) (see Ling and Bohr, 1969). As a rule the Ringer solutions were in equilibrium with a gas phase containing 95% O_2 and 5% CO_2 . In virtually all cases in which incubation lasted longer than a few hours, sterility was strictly maintained. Unless otherwise stated, the incubation solutions were gently shaken in a room maintained at a constant temperature of $25^\circ \pm 1^\circ C$.

RESULTS

Time Course of Resting Potential Change in Low and Normal K^+ Ringer Solution at 25°C

Figure 1 shows the time course of resting potential change when sartorius muscles were incubated in a Ringer solution containing not the usual 2.5 mM K^+ but virtually free of K^+ . It took about two to three hours for the resting potential to reach a new high level. If a large volume of " K^+ -free" Ringer solution (i.e., one or two muscles in 500 ml) was used, the potential having reached the high level began to fall slowly until it finally reached a new low level. When the muscle was then transferred back to a normal Ringer solution containing 2.5 mM K^+ , the resting potential would rise rapidly, approaching its original high level of 85 mV or so within 6 to 8 hours (Fig. 2). The inset of Figure 2, taken from Ling and Bohr (1970) shows the time course of changes in the total K^+ and Na^+ contents of sartorius muscles during similar exposure, first to low- K^+ and later to normal- K^+ Ringer solution. Thus the data shown in the main part of Figure 2 and in the inset represent different aspects of the same experiment: in the inset, total K^+ and Na^+ contents were recorded and in the central graph, resting potentials were recorded. Let us now compare the similarities and differences between the two.

In the low- K^+ Ringer, the time it took for the total K^+ level of the cells to fall to a final low level was about 80 hours; it also took approximately the same time (70 hours) for the resting potential to fall to the new low level.

On returning to normal K^+ Ringer, it also took nearly the same length of time (60 hours) for the total K^+ content to regain its normal value; in

sharp contrast, the resting potential required only about 6 to 8 hours to regain its normal value.

In Figure 3, return to higher potential in a normal 2.5 mM K^+ Ringer was compared in muscles that had been exposed to four different low- K^+ concentrations (0.02, 0.34, 0.60, and 0.93 mM). The time it took for the potential to return to its final high level varied with the (low) K^+ concentration to which the muscles had been exposed. At concentrations of 0.60 and 0.93 mM, the return was fast (1 hr); at concentrations of 0.02 and 0.34 mM, the return was slower (>2.5 hr).

Figure 4 shows that the return to a higher potential in normal 2.5 mM K^+ Ringer solution following exposure to low- K^+ Ringer does not depend on the size of the muscles. This independence of muscle size and hence depth of the extracellular spaces shows that the slow return to a normal resting potential is not due to a delay in diffusion through the extracellular space.

The Resting Potential at Varying External K^+ Concentrations and a Constant Na^+ Concentration of 100 mM

The fact that the resting potential of muscles exposed to low- K^+ Ringer solution required a long time to reach its new steady level shows that much of the earlier data on the effect of below-normal external K^+ concentration (normal K^+ concentration is 2.5 mM) on the levels of the resting potential, carried out after a short equilibration time, were not related to equilibrium levels; in contrast, time course studies published earlier show that the earlier data on the effect of external K^+ concentration at a K^+ concentration equal to or above 2.5 mM do represent equilibrium values (see below) (Ling, 1962; Ling and Gerard, 1950).

Figure 5 presents a plot of the steady resting potentials of frog sartorius muscles at low external K^+ concentration after 3 days' incubation at $25^{\circ}C$ and at high external K^+ after only 10 to 15 minutes of equilibration.

After resting potential measurements were made, each muscle was returned to a normal 2.5 mM K^+ Ringer solution. All muscles then regained a normal resting potential similar to that already shown in Figure 2. This precaution assures that the measured resting potentials were not from dead muscles. The inset shows a set of theoretical curves calculated from Equation 2, published before the set of experiments cited here were undertaken (Ling, 1979). The data would fit a curve with θ equal to 0.03.

DISCUSSION

The Significance of the Widely Different Time Course for Depolarization and Repolarization

In 1960 Ling showed that the resting potential of frog sartorius muscle dropped to a new low level almost instantly following the application of a Ringer solution containing 30 mM K^+ and that this low level of potential was maintained for at least 10 hours at room temperature (Ling, 1960, see also Ling, 1962), in spite of the fact that there was a steady gain of intracellular K^+ during this period of time (Ling and Ochsenfeld, 1966). This constancy of the resting potential in the face of a steadily rising internal K^+ concentration offered one early set of evidence against the membrane theory, predicting that the resting potential depends directly on the intracellular K^+ concentration.

It was also shown that a Corning 015 glass electrode is not sensitive to K^+ . Yet, application of a very thin layer of oxidized and partially dried

collodion rendered the glass electrode sensitive to the K^+ concentration in the environment (Ling, 1962; 1967a, 1967b). These findings led to the conclusion that it is not the ionic permeability through the membrane that determines the ion to which the electrode is sensitive. Rather, the selective adsorption of the surface anionic sites underlies the sensitivity exhibited by the 015 glass electrode to H^+ and not to K^+ , by the collodion-coated glass electrode to both H^+ and K^+ , or by the frog sartorius muscle to K^+ but less to Na^+ . Only a few layers of anionic sites at the surface of the living cells or their model glass electrodes determine the potential.

On the basis of this theory one can anticipate that the resting potential of living cells would change abruptly when the cells are plunged into a Ringer solution containing a K^+ concentration higher than 2.5 mM because all that is involved is the occupancy of a very small number of vacant sites at the surface. Similarly, when the muscle is plunged into a rather small volume of Ringer solution containing 0.6 to 1.0 mM of K^+ , one observes a fairly rapid attainment of a new higher level of potential. Again, this is not difficult to understand, because it involved primarily a decrease in the number of K^+ ions adsorbed at the surface. However, when the muscle is shaken in a large body of Ringer solution containing virtually no K^+ , the muscle as a whole loses K^+ slowly and steadily. In the process, the surface anionic sites will continue to receive K^+ from the inside of the cell and thus maintain a high potential until eventually intracellular K^+ is exhausted, at which time all the surface anionic sites will become occupied by Na^+ , and the potential will approach a level of nearly zero. The similar time courses for a loss of total cell K^+ and a gain of total cell Na^+ on the one hand, and for a fall of resting potential, on the other, support this view.

When a depolarized muscle is returned to a normal 2.5 mM K^+ Ringer solution, reoccupancy of the cell surface anionic sites again involves only adsorption of K^+ to the surface layer of sites and can be expected to be rapid. However, in contrast to the exposure of normal muscle to a higher than normal K^+ concentration (e.g., 30 mM), this return to normal potential involves not only simple site occupancy but also a cooperative transition of the surface anionic sites from the Na^+ state to the K^+ state. Like all similar cooperative, or more correctly, stochastic, process, this transition is time-dependent (Negendank and Karreman, 1978; Huang and Negendank, 1980), which, we believe, explains why it would still take some 6 to 8 hours for the resting potential to reach the new equilibrium value even though this is still about 8 times faster than the time required for total intracellular K^+ to return to its normal level. Indeed, according to the inset in Figure 2, one would expect that by the time the resting potential has risen to its full value, intracellular K^+ concentration could not have recovered more than a fraction of its initial concentration. These findings offer additional support for the SA theory of the cellular resting potential, which predicts that there is no direct dependence of ψ on the bulk-phase intracellular K^+ concentration but that only adsorption on the surface anionic sites determines ψ .

Autocooperativity Among Surface Anionic Sites

As mentioned in the Introduction, there is now widely confirmed evidence that cooperative interaction exists among the K^+ - and Na^+ - adsorbing sites within a variety of living cells. Recently, three laboratories using a total of four different methods (autoradiographic methods, transmission electron-microscopy, x-ray microprobe analysis, and laser microprobe mass-spectrometry (LAMMA)), all showed that K^+ in frog muscle is not free and

evenly distributed inside the cells but is adsorbed within the A bands and Z lines (Ling, 1977; Edelmann, 1977, 1978a, 1978b, 1980; Trombitas and Tigyi-Sebes, 1979). This conclusion is further supported by the observation of Huang et al (1979) of the x-ray absorption edge fine structure of K^+ in frog red blood cells. Their data strongly suggest that K^+ is in a state of complex binding. Taking all the findings together we feel that the cooperative adsorption isotherms of K^+ and Na^+ originate from interaction between intracellular K^+ -adsorbing sites that exist primarily on cell proteins, as described by the association-induction hypothesis (Ling, 1966; Ling and Bohr, 1970; Jones, 1970; Karreman, 1972; Gulati, 1973).

With this point firmly established, one recalls that for frog sartorius muscles, the average intrinsic equilibrium constant at $25^\circ C$ for the bulk-phase K^+ - and Na^+ -adsorbing sites (K_{Na-K}^{oo}) is equal to 135, and the nearest-neighbor interaction energy ($-V/2$) is equal to 0.54 Kcal/mol. The data presented in Figure 5 also permit us to estimate that K_{Na-K}^{oo} equals roughly 210 and $-V/2$ is 1.0 Kcal/mole.

The demonstration that the surface anionic sites are autocoenoperatively linked is of considerable importance. According to the AI hypothesis it is this autocoenoperativity that provides the basis for the action potential, during which the all-or-none shift of electron density (the c-value of the AI hypothesis (Ling, 1962)) of the surface anionic sites from a state of overwhelming preference for K^+ over Na^+ to one in which there is a greater preference for Na^+ occurred. Concomitant with this c-value shift, depolarization of water at the cell surface leads to the inward Na^+ current followed in turn by a return to the K^+ state (Ling, 1962, 1971, 1982, see also Ling, 1973).

The fact that during the passage of an action current there is an

increase not only in Na^+ permeability but also of uncharged molecules like erythritol and sucrose adds support to the concept that depolarization of cell surface water increases permeability to all large, complex molecules and hydrated ions during the activated state (Villegas et al, 1965).

It is also interesting to ponder the basic similarity in the values of $K_{\text{Na}^+}^{\text{OO}}$ and $-\gamma/2$ for the bulk-phase adsorption sites for K^+ and Na^+ and for the surface sites, which would suggest that similar sites are involved. We have already shown that the surface anionic sites have a pK value around 4.6, which is characteristic of the β - and γ -carboxyl groups (Ling and Ochsenfeld, 1966). Furthermore, the bulk-phase K^+ -adsorbing sites are those β - and γ -carboxyl groups concentrated in the A bands (Ling, 1977; Edelmann, 1977). The same reasoning led to the prediction that the K^+ -adsorbing sites in muscle cells are in fact the same sites that adsorb uranium ion in an EM preparation - a prediction fully confirmed by the autoradiographic study of Ling (1977) and of Edelmann by the transmission electron microscope and x-ray microprobe analysis (Edelmann, 1977, 1978a, 1978b). Thus the dark uranium-stained double-lines referred to as the unit membrane may perhaps correspond to the β - and γ -carboxyl groups concentrated at the cell surface.

HKI Theory, AI Hypothesis, and the Cellular Resting Potential When Normal External K^+ Concentration is Below Normal

A logarithmic relation between external K^+ concentration and the resting potential of isolated nerves was reported in 1900 by MacDonald (MacDonald, 1900). Curtis and Cole (1942) studied the effect of a wide range of external K^+ concentrations on the resting potential of squid axon; they noted that at external K^+ concentrations below that in the normal environment the resting potential did not continue to rise with decreasing K^+ concentration, as pre-

dicted by the Nernst equation. Instead, the potential became stabilized at a more or less constant level. Similar observation was made by Ling and Gerard (Ling and Gerard, 1950) and many others (cited in Ling, 1962).

In 1949 Hodgkin and Katz (Hodgkin and Katz, 1949) adopting Goldman's constant field theory, introduced their equation for the cellular electric potential:

$$\psi = \frac{RT}{F} \ln \frac{P_K [K^+]_{in} + P_{Na} [Na^+]_{in} + P_{Cl} [Cl^-]_{ex}}{P_K [K^+]_{ex} + P_{Na} [Na^+]_{ex} + P_{Cl} [Cl^-]_{in}}, \quad (6)$$

where P_K , P_{Na} , and P_{Cl} are the permeability constants of the cell membrane for K^+ , Na^+ , and Cl^- , respectively. $[Cl^-]_{ex}$ and $[Cl^-]_{in}$ are the extracellular and intracellular chloride ion concentrations, respectively. Other symbols are as defined earlier.

Later, this equation was modified and took the following form (Katz, 1966):

$$\psi = \frac{RT}{F} \ln \frac{P_K [K^+]_{in} + P_{Na} [Na^+]_{in}}{P_K [K^+]_{ex} + P_{Na} [Na^+]_{ex}}. \quad (7)$$

The theoretical justification for dropping the chloride terms was seriously challenged (Ling, 1978), although the experimental basis for this elimination was unquestioned (Hodgkin and Horowicz, 1959). For short-term experiments, at least, Equation 6 can be further simplified and generalized into the following form:

$$\psi = \text{Constant} - \frac{RT}{F} \ln \sum_{i=1}^n P_i [P_i^+]_{\text{ex}} \quad (8)$$

where P_i is the permeability of i th permeant cation P_i^+ . Equation 8 is identical in form to Equation 2, derived on the basis of the AI hypothesis; however, the coefficients P_i 's, on the one hand, and K_i 's, on the other, have quite different physical bases. The results of experiments to test the predictions of each model support Equation 2 but not Equation 8 (Edelmann and Baldauf, 1971; Edelmann, 1973; Ling, 1978) and will be discussed next.

As mentioned above, both theories predict a simple logarithmic relation between ψ and external K^+ concentration at or above normal concentration and can also explain the stabilization of ψ at K^+ concentration below normal, because in these experiments $[Na^+]_{\text{ex}}$ is more or less constant.

It was Weidemann who observed that in canine Purkinje muscle fibers the resting potential did not stabilize at a constant value as $[K^+]_{\text{ex}}$ continues to decrease below its normal value and as $[Na^+]_{\text{ex}}$ was held more or less constant at near its normal value in the Ringer solution (Weidemann, 1956). Instead, the potential decreased at very low external K^+ , just as we have observed and as is shown in Figure 5. This observation was later confirmed and extended by Ruzyllo and Vick in canine Purkinje muscle (Ruzyllo and Vick, 1974) and by Gorman and Marmor in molluscan neurons (Gorman and Marmor, 1970).

In the experiments reported here, a profound difference existed in the time needed for the resting potential of frog muscle to attain a new equilibrium level for muscles plunged into a Ringer solution containing more or less K^+ than that in the normal Ringer solution. For high external K^+ , the equilibrium is reached almost instantly (Ling, 1960); for low K^+ , it took many hours. In sharp contrast, it took only 15 minutes of equilibration time for Ruzyllo

and Vick (Ruzyllo and Vick, 1974) to observe the same low equilibrium potential at very low external K^+ concentration.

Earlier, we explained that the slow attainment of the new equilibrium level of the resting potential in low $[K^+]$ is probably due to slow loss of K^+ from within the cells and the continued supply of K^+ to the surface potential-determine sites. If this interpretation is correct, there can be only one interpretation for the rapid attainment in cardiac muscles of the new equilibrium level of the resting potential in low $[K^+]_{ex}$; the time needed for the loss of intracellular K^+ must be much faster. To our best knowledge, there is no exact counterpart of the data shown in the inset of Figure 2 for canine heart muscles. However, Edelmann, Pfleger, and Matt did report labeled K^+ efflux of guinea pig heart muscles; the time of 90% exchange ($t_{0.9}$) at $37^\circ C$ is about 50 minutes (Edelmann et al, 1971). In contrast, $t_{0.9}$ for labeled K^+ exchange at $25^\circ C$ in frog muscle is about 2000 minutes ($25^\circ C$)! The difference is far beyond what one would anticipate with a difference in temperature of $12^\circ C$. Thus it seems that indeed a much more rapid exchange of cell K^+ exists in mammalian cardiac muscles than in amphibian voluntary muscles, and at this level, the data support the above interpretation.

SUMMARY

The resting potentials of frog sartorius muscles equilibrated in Ringer solutions containing low K^+ concentrations were studied. Data show that surface anionic sites are responsible for the resting potential. At low external K^+ concentration and high external Na^+ concentration these surface anionic sites autocoooperatively shift to the Na^+ state in a way that is qualitatively predictable from the surface adsorption theory, a corollary of the association-induction hypothesis.

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LEGENDS

Figure 1 - Time course of depolarization of resting potential of a frog sartorius muscle in "K-free" Ringer solution. Sartorius muscles were incubated in 500 ml of a sterile Ringer solution containing only a trace of K^+ (i.e., 5 μM), and resting potentials were measured at intervals.

Figure 2 - Time course of depolarization of resting potential of a frog sartorius muscle in "K-free" Ringer solution and repolarization on subsequent return to a Ringer solution containing the normal K^+ concentration (2.5 mM) marked by the arrow. Inset is from Ling and Bohr (1970). For details of inset, see text.

Figure 3 - Recovery of resting potentials following return to a normal 2.5 mM K^+ Ringer solution after three days of prior incubation in various low- K^+ Ringer solutions; the concentrations of K^+ in these low- K^+ incubation solutions were 0.93 mM (A), 0.60 mM (B), and 0.34 mM (C) and 0.02 mM (D). Each point represents the mean \pm S.E. from at least three individual measurements.

Figure 4 - Recovery of resting potentials following return to a normal 2.5 mM K^+ Ringer solution at 25°C with gentle shaking. Each point represents a total of 16 readings from four different muscles. The average weights of the large muscles were 164.3 ± 1.3 mg; that of the small muscles (B) 93.5 ± 5.7 mg.

Figure 5 - Variation of the equilibrium resting potential of frog sartorius muscles at different external K^+ and constant external concentration. All points corresponding to an external K^+ concentration of 4 mM

or higher were obtained by the conventional procedure; measurements were made 10 minutes after the application of each higher K^+ concentration. In this range, for 4 mM to 100 mM external K^+ , each point represents the average of four single determinations. The standard errors are smaller than the width of the points and are not expressed. For points corresponding to 2.5 mM or lower concentrations of K^+ , the muscles were incubated for three days at various K^+ concentrations. After resting potentials of the muscles were placed in a normal 2.5 mM K^+ Ringer solution, to test and confirm their ability to return to normal. Each point represents average and S.E. of six readings.

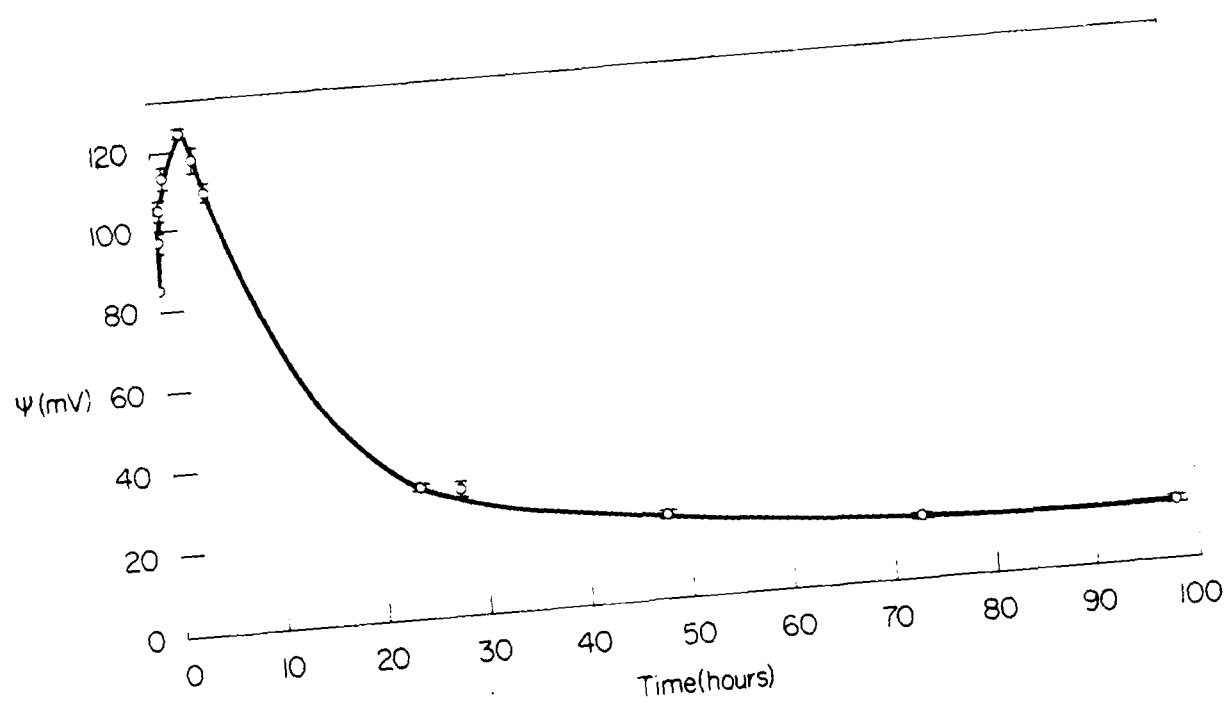


FIGURE 1

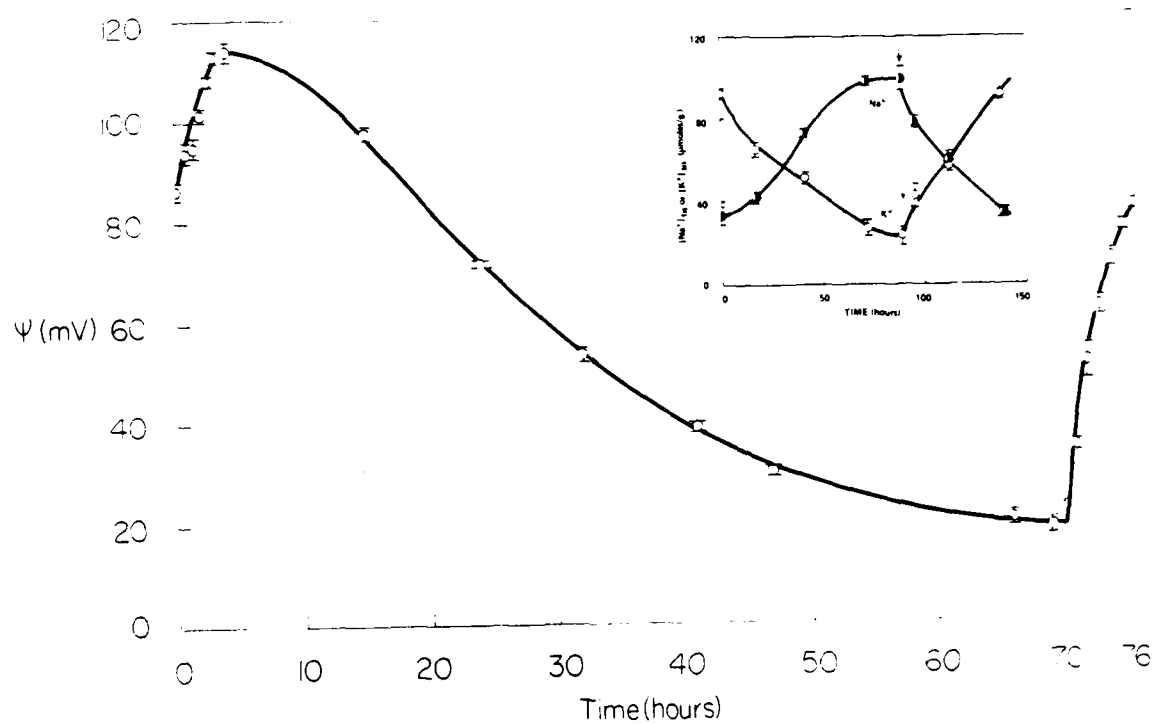


FIGURE 2

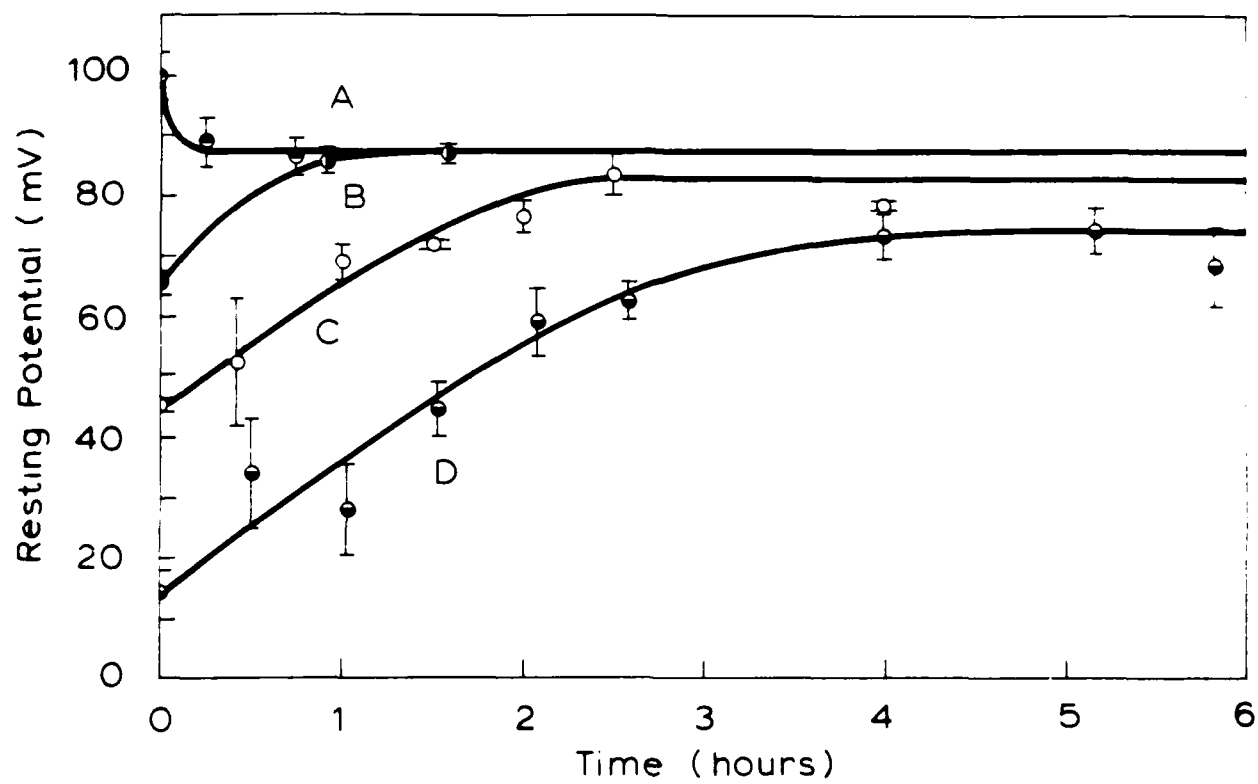


FIGURE 3

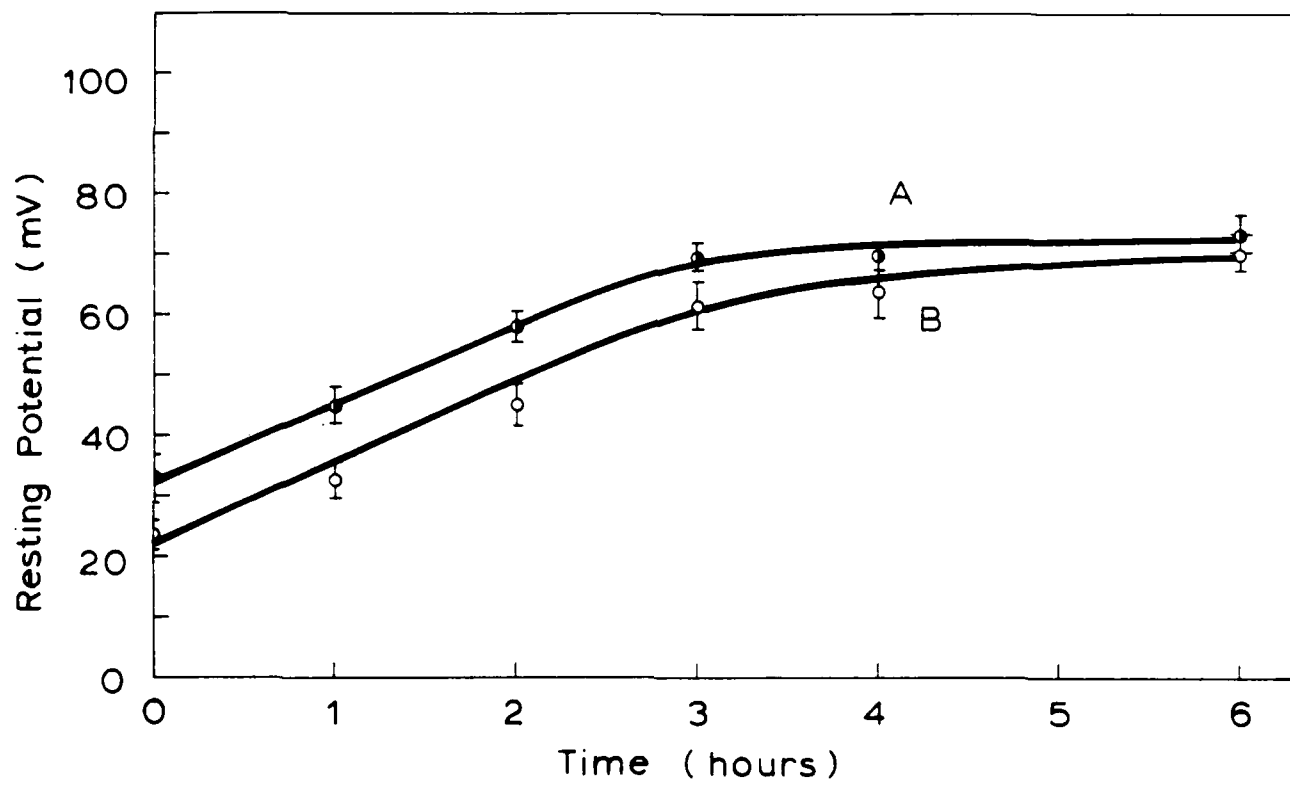


FIGURE 4

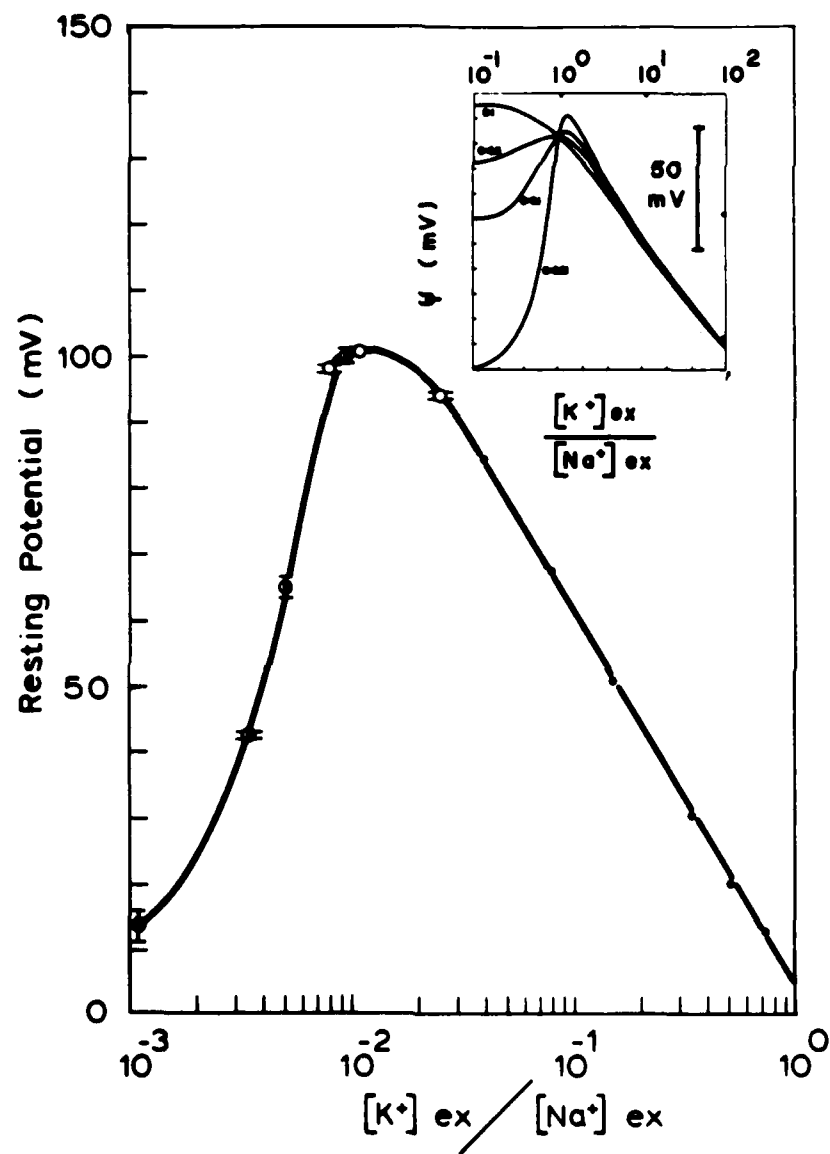


FIGURE 5

NMR RELAXATION OF WATER PROTONS UNDER THE INFLUENCE OF PROTEINS AND OTHER LINEAR POLYMERS

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The concept that water in living cells may exist in a physical state different from that of normal liquid water¹⁻⁵ stimulated considerable interest in the nuclear magnetic resonance (NMR) relaxation times of water protons in living cells,⁶⁻⁸ (for review, see ref. 9). The low T_1 and T_2 , as well as the high ratio of T_1/T_2 observed in living cells, led some scientists to the belief that the NMR data lent direct support to the association-induction hypothesis, according to which all or nearly all cell water exists in a state of polarized multilayers (PMW).

Subsequent investigations, however, led many to a different conclusion. It was argued that the short T_1 , T_2 and high T_1/T_2 ratio reflect only the properties of a minor-phase water in rapid exchange with bulk-phase water, which is simply normal liquid water.⁹⁻¹⁰

It is gratifying to note that these efforts aimed primarily at solving a basic science problem have already led to the recognition of one fundamental attribute of cancer¹¹ and the development of a potentially powerful tool for clinical medicine, the FONAR.¹² In both, Damadian played a major and critical role.

A major difficulty in using NMR relaxation time studies to determine whether or not the bulk-phase water exists in the PMW state lay in the lack of recognized criteria by means of which NMR proton relaxation times could be used to differentiate normal water and PMW. This lack of known distinguishing NMR traits of PMW in turn reflected the fact that at the time the PMW model was proposed there was no readily available

specimen of water firmly established as existing in the polarized multilayer state. Without an inanimate model, the assumption that the major phase cell water is entirely normal liquid water became the more appealing, whereas in truth the "cover-up" effect of a minor phase rapidly relaxing water is so powerful that the bulk-phase water may very well have relaxation times quite different from those of normal liquid water and still remain camouflaged.

Over the last few years, however, this laboratory has succeeded in developing a simple but efficient method of diagnosing PMW. By use of that method, the existence of PMW has been established as well as the conditions that convert normal liquid water into PMW. Specifically, the method consists of exposing water to a matrix of fairly closely placed chains containing oxygen atoms, the distances between the nearest neighboring oxygen atoms being roughly equal to twice the diameter of a water molecule.^{13,14} The number of layers of water that can be effectively polarized between chains is estimated as falling below 10 molecules between a pair of polarizing chains.

Among the water-affecting polymers studied as matrices, the most intriguing is poly (ethylene oxide) (PEO) $(-\text{CH}_2-\text{O}-\text{CH}_2)_n$ because of its extreme simplicity; this polymer has no side chains whatsoever. Other effective polymers are polyvinylmethyl ether (PVME), polyvinyl-pyrrolidone (PVP), and gelatin.

We have now carried out a series of studies of the NMR proton relaxation times of water in highly purified polymer-water systems.

83 12 21 002

Reserving the detailed data for a full presentation elsewhere, we report here the following basic findings:

(1) Both T_1 and T_2 of the polymer-oriented water are considerably shorter than those of normal liquid water, becoming shorter with increase of polymer concentrations.

(2) The ratio T_1/T_2 , on the other hand, is close to unity (1.0 to 1.4) in all concentrations of PEO, PVME, and PVP solutions; it resembles the T_1/T_2 ratio of normal liquid water at near neutral pH.¹⁵

(3) Gelatin solution like that of native bovine serum albumin, shows a much higher T_1/T_2 ratio at high polymer concentrations.

Taken as a whole, the data can be interpreted as follows: water in the dynamic state of polarized multilayers does indeed suffer rotational (and translational) motional restriction¹⁻⁵ but far less than that seen in solid ice. The correlation time, τ_c , for water effectively excluding Na^+ , sucrose, and glycine, is estimated to be no larger than 3.5×10^{-11} sec (25°C) and thus not more than 10 times slower than that in normal water. τ_c progressively decreases with decreasing water content of the polymer-water system, reaching a value of 10^{-11} sec at 20 to 35% water contents. Since the Debye dielectric rotational correlation time (τ_{rd}) is equal to $2.5 \tau_c$,¹⁶⁻¹⁸ the corresponding τ_{rd} should be 2.5×10^{-11} sec. It is interesting to compare this value with the additional dispersion of wet lysozyme powder with a τ_{rd} of 2×10^{-11} sec that Harvey and Hoekstra¹⁹ observed when the water content increased beyond 0.35 g/g protein. This water content corresponds roughly to the limit of polar group hydration.²⁰ Therefore the additional hydration referred to might be largely due to polarization by the polypeptide NHCO groups and thus would be polarized in a manner similar to polarization of the PEO-water system.^{13,14,20}

The T_1/T_2 ratios were found to be quite different in the two proteins studied. Here a minor phase of more rapidly relaxing water in rapid exchange with the major phase water

can explain the different behavior of both native bovine serum albumin solution, whose water is essentially normal as judged by its solvency for Na^+ , sucrose, and glycine, and of gelatin "gel" where water has reduced solvency for these probe molecules.¹³ □

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PRELIMINARY NOTE

APPARENT SIMILARITY IN PROTEIN COMPOSITIONS OF MAXIMALLY DEVIATED CANCER CELLS

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Using SDS gel electrophoresis, we examined the total protein contents of 14 types of mouse cancer cells (Kreb's, Reif-Allen, P815, Hepatoma 134, P4132, LSA, TA3, L1210, P1081, Ehrlich, Meth. A, 15091A, Sarcoma 180, T241) and 5 types of rat cancer cells (Walker 256, Yoshida hepatoma, Novikoff, AS30, Dunning leukemia). We then compared those contents with the cellular protein contents of normal mouse and rat tissues (brain, muscle, liver, spleen, heart, lung, nerve).

The results show, on the one hand, much similarity in the kinds and amounts of proteins from the various types of cancer cells although they derived originally from widely different tissues. On the other hand, great diversity is seen among the proteins from normal cells, as to be expected. Eight of the major polypeptide bands seen in all cancer cells studied gave apparent molecular weights of 34,000, 36,900, 46,100, 49,800, 57,000, 59,200, 69,600 and 92,500 daltons respectively. All the cancer cells were what Potter¹ calls "maximally deviated" as indicated by their very short transplantation time (i.e., one week).

Our findings, to be fully described elsewhere, extend and are in harmony with the conclusions of J. Greenstein^{2,3} from his studies of one special kind of protein, the enzymes. These he found different in normal tissues but more alike in the cancer cells he studied.

The present results suggest that cancer may indeed represent a cellular change to either a single ontologically earlier totipotent state or to a single new totipotent state. In either case, apparently actively transcribed genes specific to their parent normal tissues are shut off and a specific assembly of genes common to all cancer cells is transcribed to produce highly similar if not identical cancer cells regardless of their ancestry. □

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